Identification of *Legionella* Outer Membrane Proteins for the Development of a Biosensor

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Declaration

I certify that except where due acknowledgment has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; and, any editorial work, paid or unpaid, carried out by a third party is acknowledged.

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Date: _____

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List of abbreviations and symbols

Ω	ohms
~	approximately
µF	micro Farads (Capacitance)
µg	microgram
µL	microliter
µm	micrometer
Amp^R	ampicillin resistance
bp	base pairs
BSA	Bovine serum albumin
°C	degree Celsius
CFU	colony forming units
CTAB	cetyl-trimethyl ammonium bromide
Da	dalton
DNA	Deoxyribonucleic acid
EDTA	ethlenediaminetetraacetic acid
et al	et ali, and other people
g	gram
hr	hour
H_2O	water
H_2O_2	hydrogen peroxide
HEPES	N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilo basepairs

kDa	kilo dalton
KDO	3-Deoxy-D-*manno*-octulosonic acid
Km^R	kanamycin resistance
kV	kilo volts
λ	lambda
LBA	Luria Bertani agar
LBB	Luria Bertani Broth
LPS	lipopolysaccharide
mA	milliampere
MALDI-TOF	Matrix-assisted Laser Desorption/Ionisation- Time of Flight
MS	mass spectrometry
mg	milligram
min	minutes
ml	milliliter
mM	millimole
M	molarity
MW	molecular weight
ng	nanogram
O_2	oxygen
OMP	outer membrane protein
ORF	open-reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBT	phosphate buffered saline/Tween-20
PCR	polymerase chain reaction

pH	potential of hydrogen
RT	room temperature
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
s	seconds
spp.	species (plural)
ss.	subspecies
SSC	sodium chloride, sodium citrate buffer
TAE	tris, acetate, EDTA buffer
TE	tris, EDTA buffer
TEMED	N,N,N,N'-tetramethylethyleidiamine
Tris	tris (hydroxymethyl) amino methane
Tris·Cl	tris hydrochloride
TST	Tris-buffered saline, tween 20
U	units
UV	ultraviolet
V	volts
v/v	volume per volume
w/v	weight per volume
w/w	weight per weight
X-gal	5-bromo-4chloro-3-indoyl-β-D-galactose

Summary

Legionella spp. can cause a life threatening form of pneumonia, which is observed world-wide. Outbreaks of the disease are, unfortunately, not a rare event, despite the introduction of government regulations which enforce the mandatory testing of cooling towers to ensure that they contain levels of the organism which are regarded as being within safe limits.

Therefore, cooling towers should be monitored for *Legionella* spp. by using a biosensor. These could potentially save the community from a great deal of morbidity and mortality due to legionellosis.

This study identified and investigated novel outer membrane proteins in *L. pneumophila,* and analysed their potential for use in a *Legionella* biosensor.
A combination of bioinformatics and laboratory investigations was used to identify the Omp87, an outer membrane protein of *L. pneumophila* which had not been previously described in this organism. Sequence analysis of the protein showed that it shares similarity with various other members of the Omp85 protein family, including the D15 antigen of *Haemophilus influenzae* and the Oma87 of *Pseudomonas aeruginosa*.

The *omp87* gene of *L. pneumophila* was amplified and cloned, and was found to encode a protein of 786 amino acids, with a molecular weight of 87 kDa. Distribution studies revealed that the gene is present in most, but not all species and serogroups of *Legionella*.

To investigate the function of the Omp87 protein in *L. pneumophila*, the *omp87* gene was insertionally inactivated with the use of a kanamycin resistance gene. Amplicons of this disrupted gene were then introduced into *L. pneumophila*, and a double-cross over event occurred, integrating the inactivated gene into the genome of the organism. This resulted in non-viable cells, indicating that the gene is essential in *L. pneumophila*.

The expression vector pRSETA was used to express the Omp87 protein in *E. coli*, and four truncates of varying sizes were designed, through the use of different PCR primers. Two of the protein truncates were then expressed and purified by gravity flow chromatography using columns packed with Ni-NTA sepharose resin.

Following analysis of the proteins by SDS-PAGE and Western blotting, polyclonal antibodies were raised against the truncates. Distribution studies were then performed using the antiserum with different strains and species of *Legionella*. This study demonstrated that most serogroups of *L. pneumophila*, and most other *Legionella* species reacted with the polyclonal anti-Omp87 *L. pneumophila* antisera. Cross-reactivity was also observed with most other *Legionella* related organisms tested.

The results presented in this thesis demonstrated that the Omp87 protein or the *omp87* gene can be used to construct a biosensor. In addition other novel outer membrane proteins were identified which could also serve as potential targets for a biosensor.
These biosensors will be able to identify *Legionella* spp. in water reservoirs and in clinical samples and hopefully reduce the number of infections and deaths caused by this organism.

Chapter I

General Introduction

1. Family *Legionellaceae*

The family *Legionellaceae* belong to the gamma (γ)-proteobacteria class of the phylum Proteobacteria. The γ-proteobacteria represent this phylum's largest subgroup and although all members are phylogenetically related through 16S rRNA gene sequences, this subgroup consists of a remarkably diverse physiological range of organisms. This diversity includes chemoorganotrophs, photolithotrophes, chemolithotrophs, and methylotrophs. Genera in the class include *Legionella*, *Chromatium, Leucothrix, Pseudomonas, Azotobacter, Vibrio, Escherichia, Klebsiella, Proteus, Salmonella, Shigella, Yersinia and Haemophilus* (Prescott *et al*, 2005).

Legionella was first recognized as a human pathogen in 1976, when it was isolated from members of the American Legion attending a bicentennial celebration in Philadelphia. The organism was named *Legionella pneumophila* (Brenner *et al*, 1979) with the species name *pneumophila* coming from the Greek language for "lung-loving". Of the 182 people affected with the 'mystery' pneumonia, which in up to half of the cases was accompanied by abdominal pain and diarrhoea, almost 30 people died. The resulting media attention led to a rapid and thorough scientific investigation of the outbreak which in turn, led to the identification and characterisation of the organism (Fraser *et al*, 1977).

1.1 General characteristics

Legionella spp. are aerobic, thin, gram negative bacilli, that vary in length between 2-20 μm. Following numerous passages on solid agar culture media, long filamentous forms of the organism may occasionally develop (**see Figure 1.1**, Faine *et al,* 1979). Due to the possession of flagella (single or multiple, polar or subpolar), most species of *Legionella* are motile. Visualisation of the organism by conventional staining methods can often be difficult due to their thin peptidoglycan layer (Chandler *et al*, 1977). *Legionella* species are nutritionally fastidious and require L-cysteine and iron supplemented media for primary isolation. They are non-fermentative, nitrate negative, catalase and gelatinase positive, and do not hydrolyse urea (Murray *et al*, 2005).

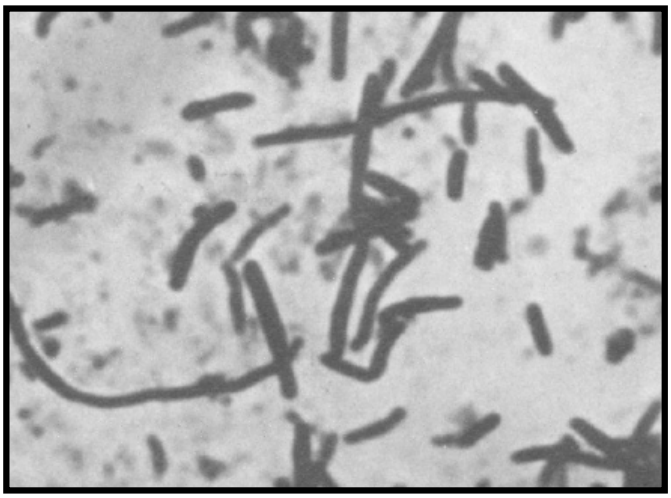


Figure 1.1 - Silver-stained smear from a culture of *L. pneumophila,* showing longer filamentous forms x 1,500 (Faine *et al*, 1979).

1.1.1 Classification of Species and Antigenic Variants

The initial isolation and molecular characterisation of a *Legionella* spp. led to the formation of the new '*Legionellaceae'* family of bacteria. The genus *Legionella* was formed, and today consists of almost 50 species, and over 70 serogroups, all of which have been categorised following studies of DNA homology (www.*Legionella*.org). *L. pneumophila* currently has 15 serogroups, which differ based on their Lipopolysaccharide (LPS) structure (Otten et al, 1986).

1.1.2 Nutritional requirements

Although *Legionella* is described as a fastidious organism, it appears that it is only when grown *in vitro* that it requires complex media for growth. Tap water, harsh environmental

conditions and phagocytic cells are all regarded as comfortable living environments for the organism (Winn and Myerowitz, 1981).

Legionella spp. derive their energy from amino acids, as opposed to carbohydrates, and this forms the basis of their main nutritional growth factor requirement, the amino acid L-cysteine. In the laboratory, the medium of choice for the culture of *Legionella* species is Buffered Charcoal Yeast Extract (BCYE) agar. This medium contains L-cysteine, yeast extract, soluble ferric (Fe^{3+}) pyrophosphate, and α-ketoglutarate. It also contains charcoal, which absorbs and inactivates toxic peroxides that develop as a byproduct of *Legionella* growth. Finally, a buffer is also included which maintains the pH of the medium at 6.9, which is ideal for *Legionella* spp. growth (Murray *et al*, 2005).

1.1.3 Laboratory Diagnosis

The rapid and successful identification of *Legionella* spp. as the causative agent of disease is paramount. Often, cases of Legionnaires' disease are epidemic in size, and numerous individuals are involved. This adds to the concern of laboratory staff identifying the organism, particularly if there are elderly and high risk patients involved. Antibiotic therapy must be initiated as soon as practically possible, in order to improve the success rate of therapy, and reduce the risk of fatalities. Since the discovery of *Legionella*, a vast number of techniques have been developed and assessed for the identification of *L. pneumophila* and other *Legionella* species. Older techniques include microagglutination, immune adherence haemagglutination, indirect haemagglutination, indirect immunofluorescence and ELISA (Herbrink *et al*, 1983). More recent methods for *Legionella* spp. detection are mostly based on molecular identification, and revolve around PCR-based techniques such as real-time, nested and multiplex PCR and hybridisation reactions. Some of these techniques will be reviewed in more detail here.

1.1.3.1 Conventional diagnostic methods

1.1.3.1.1 Culture

Considered the 'gold-standard' for diagnosis, culturing of *Legionella* spp. is routinely used by laboratories as an effective screening method for the presence of *Legionella* spp. in both clinical and environmental samples (Murray *et al*, 2005). The standard non-selective agar used for *Legionella* spp. is the agar discussed above (section 1.1.2), Buffered Charcoal Yeast Extract (BCYE) agar. Since respiratory samples are often excessively contaminated with normal flora, antibiotic supplements are usually added to the medium. Constituents added include polymyxin, anisomycin and cefamandole or vancomycin. These agents control the unwanted growth of gram negative bacteria, yeast, and gram positive bacteria, respectively. *Legionella* colonies growing on BCYE agar usually appear as round or flat colonies with smooth edges. They are usually colourless to iridescent, and may be translucent or speckled (Salyers and Whitt, 2002) **(Figure 1.2)**.

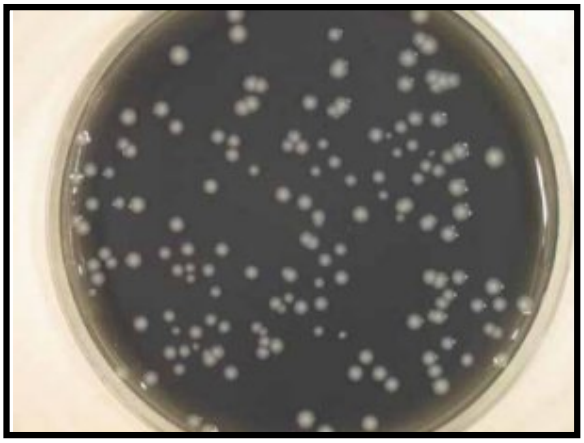


Figure 1.2 - *L. pneumophila* colonies growing on BCYE agar (www.rivm.nl).

1.1.3.1.2 Agglutination-based Assays

Several years following the discovery of *Legionella* spp., agglutination tests became available that were able to detect *L. pneumophila* serogroups. These simple tests are still used routinely in laboratories, usually in conjunction with culture methods.

Tang *et al* (1982) was one of the early groups to develop an agglutination based immunoassay for the detection of *L. pneumophila*. They developed a reverse, passive agglutination test, which was able to detect the soluble antigens of *L. pneumophila* in urine samples. Although the group described the method as rapid and simple, they did observe intra-genus cross-reactivity with several *L. pneumophila* serogroups. The group also observed that the assay did not detect one of the fifteen antigenuric samples included in the testing, which were all from patients with clinically diagnosed Legionnaires' disease.

A similar assay was developed by Sedgwick and Tilton (1983) using latex agglutination to detect *L. pneumophila.* The difference here was that the assay was designed to detect *L. pneumophila* colonies growing on agar plates, as opposed to detecting *L. pneumophila* antigens in the urine of infected patients. Here, the *L. pneumophila* samples were grown on CYE agar plates for two days at 35°C. The colonies were harvested and suspended in phospate buffered saline (PBS), and the turbidity was adjusted to 10^8 CFU per ml. One drop of this sample was then mixed with one drop of antibody coated latex particles. The mixture was then rotated and observed for agglutination. The group claimed that the assay could clearly distinguish between *L. pneumophila* serogroups 1-6, and did not react with non-*Legionella* strains included in the testing.

As with all agglutination tests, however, there is the associated difficulty of observing reactions, and objectively judging whether results are positive or negative. Often a 'scale' of agglutination is used, or the reaction time is taken into account (Mims *et al*, 2004).

1.1.3.1.3 Immunofluorescence Assays

Immunofluorescent techniques were also developed for the detection of *Legionella* spp. These assays are relatively simple to perform, and remain much in use in today's laboratories, and are usually performed in conjunction with culture **(see Figure 1.3)**.

Herbrink *et al* (1983) developed an immunofluorescence assay shortly after the discovery of the *Legionella* bacterium. The assay was based on the detection of antibody to *L. pneumophila*. The group developed a polyvalent Enzyme Linked Immunosorbent Assay (ELISA) which allowed for the rapid screening and detection of *L. pneumophila* serogroups 1-6 in human serum. The assay was designed using microtitre plates coated with the *L. pneumophila* serogroups. The group also tested the sensitivity of the assay, and found that it was comparable to that of the more laborious and time consuming technique of indirect immunofluorescence (IFA).

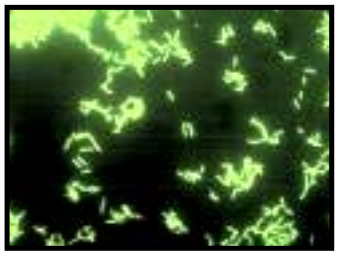


Figure 1.3 - Fluorescent image of *L. pneumophila* cells (www.hsudruga.hr).

Another technique that was evaluated for its potential as a diagnostic tool for *L. pneumophila* detection was a solid surface immunoassay that was developed by Vogel *et al* (1981). This assay involved the application of *L. pneumophila* antigen to a solid surface, and quantitation of binding through immunofluorescence. This technique employed the use of a semi-automated fluoroimmunoassay system. The group found that the procedure was able to readily detect specific *L. pneumophila* antibody in trials performed using sera obtained from *L. pneumophila* infected guinea pigs. They also found that results obtained with the fluoroimmunoassay system correlated well with those of the microagglutination assay previously developed by Farshy *et al* (1978). The group used this microagglutination assay for comparison due to its technical simplicity. Following the evaluation, Vogel *et al* concluded that the results of the fluoroimmunoassay were comparative to those of the

microagglutination assay, yet that this assay was much quicker to perform than the microagglutination assay.

1.1.3.2 Current diagnostic methods

Culture is still the gold standard for detection of *Legionella* spp. Numerous other tests are performed in combination with culture, to confirm the finding, or to type and determine the species, strain or serogroup of the organism. These additional tests include detection of bacterial antigens, through fluorescent antibody staining, clinical testing for antigens and antibodies in urine or serum, and gene probes and PCR, for detection or amplification of bacterial DNA (Mims *et al*, 2004). More recent techniques are being continuously evaluated, however routine diagnostic laboratories often have limitations in regards to equipment, expertise, and finance.

One of the most commonly used diagnostic methods for the detection of *L. pneumophila* is urinary antigen detection (Fields *et al*, 2002). Several kits are commercially available which are similar in design and function.

Benson *et al* (2000) evaluated two commercially available urinary antigen detection kits, the Binax and Biotest kits. Although both kits were found to be highly specific, the group found that the Biotest enzyme-linked immunosorbent assay (EIA) was slightly more sensitive than the Binax EIA kit.

More recently, Okada *et al* (2002) evaluated the Biotest EIA with a newly developed Binax kit, Binax NOW. The group wanted to determine how the kits performed when presented with samples of non-serogroup 1 *L. pneumophila*, and other *Legionella* species. They used *in-vitro* extracted antigens of 22 *L. pneumophila* serogroup 1-15 strains and of 27 other *Legionella* species. They found that there was no cross-reactivity between *L. pneumophila* and other *Legionella* species, but that reactivities were different with samples of non-serogroup 1 *L. pneumophila*. The observed sensitivity of both tests with *L. pneumophila* serogroup 1 was described as excellent, but the group was unable to conclude on the sensitivity of the tests with other *L. pneumophila* serogroups.

No doubt due to the recent introduction of regulatory demands for routine testing of water cooling towers and the like, several groups have designed methods for the detection of *Legionella* spp. in water samples.

Delgado-Viscogliosi *et al* (2005) developed a method based on epifluorescence microscopy, for the enumeration of *L. pneumophila* and other *Legionella* spp. in water samples. The method, based on double-staining fluorescent labelling, uses a bacterial viability marker, and can therefore also distinguish between viable and non-viable *Legionella* spp. The group discussed the fact that although many PCR based methods for detecting *Legionella* spp. have been developed, none of these methods take into account the fact that it is only viable cells of *Legionella* spp. in water cooling systems which pose a threat to the community. The group concluded that this method was rapid and effective, and allowed users to monitor the efficiency of disinfection treatments.

Some other methods which are being developed and evaluated for *Legionella* spp. identification include the work of Yanez *et al* (2005) who developed an immunomagnetic purification and real-time PCR method for amplification of the *dotA* gene, to quantitatively detect *L. pneumophila* in water samples.

Similarly, the group of Fiume *et al* (2005) recently developed a species specific real-time and nested PCR reaction, for detection of *L. pneumophila* in water. The group targeted the *mip* gene for amplification and found that the nested PCR, in particular, was significantly more sensitive than culture in detecting organisms from water samples. They stated that the nested PCR did not require specific instrumentation, displayed a high sensitivity rate, and was therefore more valuable than either the real-time PCR assay, or cultural isolation for the monitoring and risk assessment of water samples.

1.2 Pathogenesis of Legionellosis

In the environment, *L. pneumophila* organisms survive and replicate within their natural host, amoebae. It is widely believed that amoebae have helped *L. pneumophila* to evolve and be capable of surviving within human alveolar macrophages, and therefore cause infection in humans (Rowbotham, 1980). *L. pneumophila* can infect more than thirteen species of amoebae, and are especially prevalent in the soil and water genera, *Acanthamoeba* and *Naegleria* (Fields, 1996). Adding to the credibility of this evolutionary theory is the fact that *L. pneumophila* interacts with host cell organelles in both mammalian and protozoan phagocytes in an almost identical manner (Swanson and Hammer, 2000).

Human hosts usually acquire *L. pneumophila* infections by the inhalation of contaminated aerosols (Muder *et al*, 1986). The sources of these aerosols are diverse, and include air-conditioning systems, clinical respiratory devices, whirlpools, showers, fountains, and mist machines (Barbaree *et al*, 1986; Breiman *et al*, 1990; Hlady *et al*, 1993; Jernigan *et al*, 1996; Kioski *et al*, 1997; Muder *et al*, 1986). If the droplet size of these aerosols is below around 5 microns the organisms may then travel down to the lower respiratory tract, where infection is initiated by the engulfment of cells by alveolar macrophages in the lungs (Bollin *et al*, 1985). *Legionella* is described as an opportunistic pathogen, as in healthy people the infection is usually self-limiting, and those infected may remain asymptomatic (Salyers and Whitt, 2002). For example, many of the hotel employees who were present during the 1976 outbreak were found to be seropositive for *L. pneumophila*, yet did not display any symptoms, and did not develop Legionnaires' disease (Fraser *et al*, 1977). However, if the individual has underlying factors that impair normal host defence mechanisms which normally protect the lungs, they may become seriously ill. These underlying factors include immunosupression, advanced age, smoking, emphysema or other chronic lung diseases and hematological malignancies (Carratala *et al*, 1994).

L. pneumophila is engulfed by phagocytic cells, such as macrophages and amoebae, within plasma membrane coils, by the methods of both conventional and coiling phagocytosis **(Figure 1.4)**. In coiling phagocytosis, the cell extends a long pseudopod coil around the bacterium, and draws the bacterium into the interior of the cell. This method of phagocytosis has been observed by several other organisms, including *Leishmania* spp. and *Borrelia* spp., several spirocha etes, yeasts and trypanosomatids (Chang *et al*, 1979; Rittig *et al*, 1998).

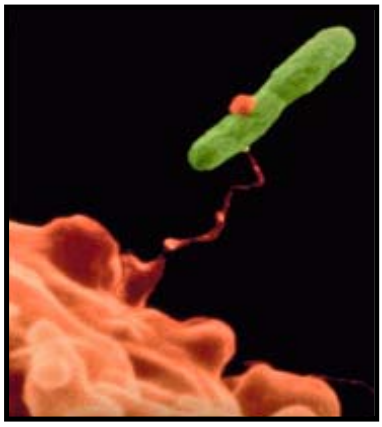


Figure 1.4 - Phagocytic engulfment of a *Legionella* bacterial cell by coiling phagocytosis (http://procareh2o.com/images/*Legionella*.jpg)

Normally, cells are engulfed by phagocytes and remain within the phagosomal compartment. This phagosome then fuses with a lysosomal compartment which leads to acidification of the phagolysosome, and bacterial cell death. However, in the case of *L. pneumophila*, this does not occur, and these bacteria seem to have the unique ability of preventing phagosome-lysosome fusion (Swanson and Hammer, 2000). The phagosomal compartment housing the *L. pneumophila* does not fuse with either primary or secondary monocytic lysosomes, and the vacuole does not acidify. Instead, the membrane bound bacterium associates with the endoplasmic reticulum, which is studded with ribosomes (Horwitz, 1983b). The *L. pneumophila* cells then replicate to large numbers, and eventually escape from the vacuole. The cells then continue to replicate until they fill the cytoplasm of the macrophage. By this stage, the bacteria begin to suffer from insufficient nutrients. The *Legionella* will then start to express traits which will soon aid in the dispersion and infection process, including piliation, motility and cytotoxicity. After this, the bacteria lyse the cell, and move on and infect other nearby cells (Swanson and Hammer, 2000). *Legionella* spp. lyse cells by a two-step process. They firstly trigger apoptosis of the cell, and then release their own cytolysins that cause cell lysis (Prescott, 2005). The genes expressed by *L. pneumophila,* which are believed to assist the organism during the macrophage invasion process, will be discussed in section 1.2.2.

Infection of the lungs by *L. pneumophila* is very destructive to lung cells. It often results in alveolitis and broncheolitis, and is characterised by an excessive amount of liquid accumulation in the alveoli, which consists predominantly of lysed lung cells. In addition this exudate also contains polymorphonuclear cells and macrophages, fibrin, red blood cells, proteinaceous material, and other cellular debris (Winn and Myerowitz, 1981). In macrophages, the *L. pneumophila* organisms can often be located intracellularly, intact, and within cytoplasmic vacuoles. Others are found free in the cytoplasm, or in membrane-bound structures (Glavin *et al*, 1979). Interestingly, a study found that most *Legionella* cells found within neutrophils were partially degraded, and had disrupted membranes (Katz and Hashemi, 1982).

It is not yet clear if the cell damage caused by *Legionella* spp. is due to the secretion of toxins or degradative products. They are known to secrete various extracellular proteases, such as the 40 kDa zinc metalloprotease, which has haemolytic activity, or phospholipases A and C. The zinc metalloprotease was found to inhibit neutrophil and monocyte killing of *Listeria monocytogenes,* and was involved in neutrophil chemotaxis (Rechnitzer and Kharazmi, 1992). When tested in guinea pigs, the *L. pneumophila* zinc metalloprotease was also found to be capable of causing the type of lung damage seen with human legionellosis (Conlan *et al*, 1986).

The phospholipases A and C may damage alveolar cells by destroying the lung surfactant, which coats cells, leading to the collapse of the alveoli cells when the gas they retain is released, due to high surface tension (Prescott *et al*, 2005).

Legionella is also known to produce a cytotoxin, called legiolysin, which was found to confer haemolytic activity (Wintermeyer *et al*, 1991). The role of this 39 kDa protein in *Legionella* pathogenesis is still unclear since a mutant strain of *Legionella,* defective in legiolysin production, still replicated efficiently in the macrophage-like cell line, U-937 cells and protozoa, and still remained haemolytic (Wintermeyer *et al*, 1994).

Toxins and secreted extracellular proteases of *Legionella* will be discussed further under the topic of *Legionella* virulence factors, in section 1.2.2.6.

1.2.1 Host immune response

With legionellosis, it has become apparent that cell-mediated immunity is the critical host immune response to infection (Friedman *et al*, 1998). In early studies performed by Horwitz (1983), peripheral blood monocytes from patients who had recovered from legionellosis were compared to control cells. It was observed that proliferation of mononuclear cells from the pre-exposed patients was much greater than that of the controls. It was also noted that the supernatants obtained from cultured mononuclear cells of these pre-exposed patients were able to activate naïve mononuclear cells, which led them to inhibit *L. pneumophila* replication. The activation of human macrophages by cytokines such as interferon-γ (IFN- γ) appears to be the critical element in the prevention of *Legionella* spp. lung infections. Bhardwaj *et al* (1986) demonstrated that when cultures of human peripheral blood monocytes or alveolar macrophages, which normally support *L. pneumophila* replication, are treated with recombinant IFN-γ for one hour, the activated phagocytes no longer support *L. pneumophila* growth, and begin to inhibit the intracellular replication of the organism.

Instead of enhancing bacterial killing, or preventing the formation of replication vacuoles, the activated macrophages are believed to starve the intracellular organisms of iron, by down-regulating their cellular transferrin receptors (Bhardwaj *et al*, 1986).

It has been well documented that *L. pneumophila* intracellular and extracellular growth is highly dependent on the organisms ability to acquire and assimilate iron (Bortner *et al*, 1989, Pope *et al*, 1996).

This observation of transferrin receptor downregulation was made by Byrd and Horwitz (1989), who noted that the ability of IFN-γ activated macrophages to inhibit *L. pneumophila* replication was reversed if the cultures were supplemented with iron. Also, Byrne and Swanson (1998) showed that IFN-γ activated blood monocytes expressed 73% less transferrin-binding sites than non-activated control cells.

Although *L. pneumophila* readily binds complement component C3, it is resistant to innate and humoral immune responses, and is resistant to complement-mediated cell killing. This was demonstrated to be the case even with the involvement of specific antibodies. Horwitz and Silverstein (1981) showed that when *L. pneumophila* was opsonised by treatment with complement and specific antibodies, the bacteria interacted proficiently with polymorphonuclear (PMN) cells, but they were not killed by the PMNs. It was shown that

under some conditions, *L. pneumophila* may prevent death by phagocytic host cells by impairing the phagocytes oxidative killing response. Lochner *et al* (1985) showed that a *L. pneumophila* toxin, which was purified from culture supernatant, had the effect of inhibiting PMN cell killing of the otherwise susceptible bacteria, *E. coli*.

Overall it has been determined that the clearing of legionellosis is not facilitated by complement, specific antibodies, or polymorphonuclear cells, but is reliant upon the involvement of the hosts cell mediated immunity.

1.2.2 Virulence factors and their Regulation

A detailed review of *Legionella* outer membrane proteins is presented in section 1.5. As some of these outer membrane proteins are also important for *Legionella* spp. virulence, please refer to section 1.5 when directed, for a more thorough review and discussion.

1.2.2.1 Macrophage Invasion Potentiator (Mip)

The *Legionella* Mip protein is a ~ 24 kDa outer membrane antigen, which displays peptidylprolyl *cis:trans* isomerase (PPIase) activity. This protein is believed to be an important *Legionella* virulence factor, which is involved in cellular invasion. This protein is discussed in detail in section 1.5, *Legionella* outer membrane proteins.

1.2.2.2 Acquisition and assimilation of iron

As mentioned briefly in sections 1.1.2 and 1.2.1, *Legionella* requires iron for growth. This has been shown by a variety of experimental studies. Firstly, *L. pneumophila* grown in monocytes which have been treated with iron chelators, do not actively replicate. This observation can be reversed by the addition of iron to the medium (Byrd and Horwitz, 1991). Secondly, it has been shown that macrophages activated by IFN-γ become non-permissive for *L. pneumophila* replication by reducing intracellular iron levels (Byrd and Horwitz, 1989). Finally, Gebran *et al* (1994) showed that following the addition of iron to the media, peritoneal macrophages from A/J mice become permissive for *L. pneumophila* growth.

By growing cells on bacteriological media, it has been determined that the amount of ferric or ferrous iron required for minimal growth is between 3-13 μM, whilst greater than 20 μM is required for optimal growth (Johnson *et al*, 1991). It has been postulated that the reason for this high requirement for iron may be due to a high concentration of an iron-containing aconitase in the cytoplasm of *Legionella* (Mengaud and Horwitz, 1993).

Unfortunately, not a great deal is known about how *Legionella* spp. acquire and assimilate iron. It is unclear whether *Legionella* spp. use the same mechanisms commonly employed by other organisms, such as siderophore production or the binding of transferrin (Johnson *et al*, 1991, Reeves *et al*, 1983). However, under specific growth conditions, Liles *et al* (Liles *et al*, 2000) demonstrated that *L. pneumophila* does possess a non-hydroxamate non-phenolate siderophore, termed legiobactin. And in addition to this, the group of Hickey and Cianciotto (1997) also found that the *L. pneumophila* genome encoded a homolog of a hydroxamate biosynthetic gene, which when disrupted, resulted in impaired growth of the organism within macrophages. This suggests that within host cells, *L. pneumophila* may produce and require an addition siderophore.

Hemin is an important component of haemoglobin, and hemin-binding is one mechanism that has been investigated since the discovery of a hemin binding protein (*hbp*) gene locus in *Legionella* (O'Connell *et al*, 1996). In addition, a transcriptional regulator, Fur, which responds to iron levels was discovered (Hickey and Cianciotto, 1994).

Viswanathan *et al* (2000) recently analysed a gene locus in *L. pneumophila* described as *iraAB*, which they identified as being responsible for the ability of *L. pneumophila* to assimilate iron. The group analysed an *iraAB* gene mutant, and found that the mutant was

defective for growth in the macrophage-like cell line, U-937. They found that even when the amount of cells inoculated was increased 50-times, the mutant failed to display any signs of infectivity. The group described the *iraAB* locus as a two gene operon. They also performed distribution studies and found that the *iraAB* genes are present in all serogroups of *L. pneumophila*, and are also present in the species *L. gormanii*, a relatively rare, but disease causing species. By creating several mutants, the group found that the role of *iraAB* in growth and pathogenesis of the organism was quite complex, and they believed that the iraA protein was essentially involved in intracellular growth, and that this was directly associated with iron acquisition. They then found that the *iraB* gene product was involved in the *extracellular* growth of the organism, and again, that this was related to iron acquisition.

Sequence analysis performed on the genes revealed that the *iraA* gene was dissimilar to most other iron assimilation genes. A BLAST analysis did reveal a strong match with the phosphatidylethanolamine methyltranserase enzyme, from *Acetobacter aceti*. This enzyme is involved in the conversion of phosphatidylethanolamine to phosphatidylcholine (PC). Although this compound is rarely produced by organisms, *L. pneumophila* cells have been found to contain unusually large amounts of PC (Finnerty *et al*, 1979).

An interesting observation was made by the group in regards to the mechanism used for extracellular iron acquisition by *L. pneumophila* with the *iraB* gene product. The group speculates that this very unique mechanism may be based on the PTR2 family of peptide transporters, whereby the organism uses the IraB protein to import iron-loaded peptides as a method for acquiring iron. Although siderophores are made up of peptide components (Dreschsel and Jung, 1998) this case may be the first real scenario whereby free peptides are used for iron acquisition.

1.2.2.3 Flagella

The flagella of *Legionella* are an important virulence factor which aids *Legionella* spp. in motility and dissemination to new host cells during infection (Pruckler *et al*, 1995). This outer membrane structure is discussed in detail in section 1.5.

1.2.2.4 Dot/Icm Type IV Secretion System

Legionella spp. are among several organisms which makes use of type IV secretion systems as an implement for added virulence. Type IV secretion systems are encoded chromosomally at loci which are involved with the conjugal transfer of plasmid DNA, and the ability of cells to transfer DNA was once the criteria identifying members of the type IV transport system pathway. In *Legionella*, the type IV secretion system is encoded by 24 genes, which are located on the bacterial chromosome at two different regions. Fourteen of these genes are the *dot* (defective for organelle trafficking) and *icm* (intracellular multiplication) genes (Andrews *et al*, 1998; Brand *et al*, 1994).

Whilst most of the *icm/dot* gene products are believed to be membrane-associated proteins, DotA is a cytoplasmic membrane protein, and IcmW is a small cytoplasmic, soluble protein.

It is believed that the Dot/Icm proteins assemble and stimulate a membrane-based secretory system in *L. pneumophila,* which is responsible for secreting virulence factors. In addition, the group of Segal *et al* (1999) recognised a second related system designated LVh (*Legionella vir* homologs) which is also involved in the transfer of plasmids by conjugation.

The major role played by the Dot/Icm type IV secretion system is the avoidance of the endocytic pathway during phagocytosis. Dot/Icm mutants all succumb to the endosomal pathway within a very short time following infection (Segal and Shuman, 1997; Wiater *et al*, 1998). This indicates that *L. pneumophila* has a very short time frame in which to alter its phagosome following infection.

L. pneumophila utilises the Dot/Icm system to create a protected vacuole, but then no longer needs the system to maintain itself within the vacuole and replicate (Roy *et al*, 1998). This is characteristic of *L. pneumophila* cells in the exponential-phase of growth, where the expression of virulence traits is often down regulated (Byrne and Swanson, 1998).

By comparing this to other type IV systems, it is hypothesised that the Dot/Icm complex of *L. pneumophila* must insert pores into the plasma membrane of the host cell vacuole, which can impair the process of phagosome maturation (Kirby and Isberg, 1998). It has been shown that Dot/Icm mutants lack this pore-forming activity, and are therefore not cytotoxic. The group of Kirby and Isberg (1998) also hypothesised that if a large number of pores are created in the host plasma membrane, the phagocyte can be rapidly lysed, in a contact-dependant process. The toxins responsible for forming the pores are yet to be discovered,

making it difficult to establish the details of this process. Zuckman *et al* (1999) proposed the theory that the pores serve as a channel through which the effector molecules can be delivered, which then modify and alter the nascent phagosomal membrane. The group suggested that the small soluble protein IcmW may be one such effector, but that instead of acting as a substrate for the type IV secretion system, it may act by the direct or indirect regulation of Dot/Icm activity. They created an *icmW* mutant, and found that although pore formation was critical to establishing an isolated phagosome, it alone was not sufficient, and the IcmW protein product was required for full virulence.

Joshi *et al* (2001) showed that Dot-independent factors are also involved in impeding phagosome maturation. They used a series of fluorescence microscopy assays to determine which bacterial factors participate in establishing a replicative vacuole, and whether *L. pneumophila* is isolated from the endosomal pathway or is contained within an intermediate endosomal compartment.

The group found that vacuoles containing post-exponential (PE) phase *L. pneumophila* appeared to be separate from the endosomal pathway, as they lacked certain categorical markers. These include the transferrin receptors **L**ysosome **A**ssociated **M**embrane **P**rotein-1 (LAMP-1) and cathepsin D.

LAMP-1 is a membrane glycoprotein, found mainly in late endosomes and lysosomes. It is believed to protect the *Legionella* membrane from the acidic and proteolytic components of the vacuole (Joshi *et al*, 2001).

In contrast, the group found that most (70%) of the phagosomes that contained avirulent particles, such as polystyrene beads, *E. coli*, or exponential phase (E) *L. pneumophila* matured to phagolysosomes, as determined by the presence of molecular markers, such as LAMP-1 and cathepsin D. This indicated that neither the Dot/Icm complex, or bacterial viability or virulence were critically required for the development of an isolated vacuole.

Recently, Molofsky *et al* (2005) established the involvement of the flagella regulon, sigma factor FliA, in the avoidance of lysosomal degradation. The group used murine bone marrow-derived macrophages (BMM) to demonstrate that in addition to coordinating motility, the FliA sigma factor also contributes to the inhibition of phagosome maturation, and hence virulence.

Miyake *et al* (2005) described a gene locus consisting of 11 *dot*/*icm* genes, designated the **p**rotozoan and **m**acrophage **i**nfectivity (*pmi*) locus. The group mutated a gene from the *pmi* locus, and found that the mutant was defective for cytopathogenicity of protozoa and

macrophages. The mutant also exhibited a partial defect for growth within U-937 cells, and a severe defect for growth within the amoeba *Acanthamoeba polyphaga*, which ultimately resulted in its elimination.

Santic *et al* (2005) recently made an interesting discovery in regards to interferon-gamma (IFN-γ) activated macrophages. It is well documented that IFN-γ activated macrophages are able to inhibit intracellular replication of *L. pneumophila*, and other intracellular pathogens (Bhardwaj *et al*, 1986, Byrd and Horwitz, 1989, 1991, 1993). By analysing the kinetic distribution of molecular markers such as LAMP-2, cathepsin D and the lysosomal tracer Texas red ovalbumin, it has been shown that IFN-γ activated macrophages are able to 'override' the mechanisms used by *L. pneumophila* to avoid phagolysosomal fusion (Santic *et al*, 2005).

Overall, it has been determined that the Dot/Icm complex is critical for the avoidance of phagosomal-lysosomal fusion of *L. pneumophila,* and for the establishment of an isolated replicative vacuole (Vogel and Isberg, 1999). Effector molecules/proteins secreted through the Dot/Icm pore channel are believed to play a central role in altering host cell activity. Effector molecules believed to be involved include DotA, DotB, DotH, DotO, IcmQ, IcmR, IcmS, IcmW IcmX and FliA (Coers *et al*, 2000; Swanson and Isberg, 1996; Matthews and Roy, 2000; Molofsky and Swanson, 2005; Watarai *et al*, 2001; Zuckman *et al*, 1999). Proteins determined to be substrates of the Dot/Icm complex have also been identified, and include LepAB, LidA, RalF, and SidC. These proteins are believed to modulate host signal transductions in the establishment of replicative vacuoles (Chen *et al*, 2004., Conover *et al*, 2003., Luo and Isberg, 2004., Nagai *et al*, 2002).

1.2.2.5 Pili

Pili, or fimbriae are short, hair like appendages which are usually involved in facilitating bacterial attachment to host cells or solid surfaces. This important *Legionella* virulence mechanism in *Legionella* spp. is discussed in detail in section 1.5.

1.2.2.6 Toxins

Not a great deal has been elucidated in regards to *Legionella* toxins and their role in pathogenesis. Several toxins and secreted compounds have been described for *Legionella* spp., but their precise role remains unclear.

One of the earliest cytotoxic peptides described in *Legionella* is a heat stable peptide, which was found to diminish the oxidative burst capabilities of polymorphonuclear cells (PMNs) (Friedman *et al*, 1980). The difficulty observed in purifying the toxin, or analysing its encoding genes, has meant that the toxin has never been fully analysed.

Legionella uses a type II secretion mechanism to secrete a variety of toxic compounds. These include acid phosphatases, a zinc metalloprotease, phospholipases A and C and a lysophospholipase A (Aragon *et al*, 2001; Flieger *et al*, 2000, 2001) If genes encoding this type II secretion process are disrupted, such as the *isp* genes, there is a significant reduction in *Legionella* virulence (Liles *et al*, 1998).

The zinc metalloprotease plays a relatively small role in *Legionella* virulence. It promotes cytotoxicity by inhibiting chemotaxis, and the oxidative activity of PMNs. It also breaks down a variety of compounds including interleukin -2 (IL-2) and CD4 (Conlan *et al*, 1988., Mintz *et al*, 1993). As mentioned in section 1.2, *Legionella* also produce a 39 kDa cytotoxin, termed legiolysin, which was found to confer haemolytic activity to recombinant *E. coli* (Wintermeyer *et al*, 1991). The role of this cytotoxin in *Legionella* pathogenesis is not yet clear.

1.2.2.7 Regulation of Virulence Factors

1.2.2.7.1 Temperature

The ambient temperature experienced by a bacterium is often used to control and regulate the expression of virulence factors. This is usually because the temperature is a useful indication of the environment in which the bacteria are residing at that time. Factors mainly influenced by temperature include motility and piliation. *Legionella* express more flagellin genes when they are incubated at 30°C, rather than 37°C (Heuner *et al*, 1999). Similarly, expression of

the *pilBCD* genes and production of type IV pili are greater at 30°C than 37°C (Liles *et al*, 1998).

Adherence of *L. pneumophila* to host cells was also found to be temperature dependent, and the organism was found to adhere to alveolar macrophages at a rate of two-fold more if the cells were incubated at 25°C, as opposed to 41°C, for 1 hr before infection (Edelstein *et al*, 1987). However, Mauchline *et al* (1994) found that according to their 50% lethal dose (LD_{50}), *L. pneumophila* cultured at the lower temperature of 24°C were generally less virulent than bacteria cultured at 37°C.

1.2.2.7.2 Growth Phase

It is well documented that in addition to temperature, the growth phase of the organism has a dramatic influence over the regulation and expression of virulence traits. One notable effect is the phenotypic difference observed between *Legionella* grown on laboratory medium as opposed to those grown within phagocytic vacuoles. *Legionella* grown within phagocytes are shorter, thicker and more motile. They have been shown to express different genes and proteins, have a higher β-hydroxybutyrate content, and different staining properties, probably due to their thicker and smoother cell wall (Abu Kwaik *et al*, 1993; Cirillo *et al*, 1999; Edelstein *et al*, 1999). Also, bacterial cells grown within amoebae were found to have a different profile of membrane fatty acids, LPS, and outer membrane proteins, than cells grown in broth. They were also more susceptible to the protein degrading enzyme, proteinase K (Barker *et al*, 1993).

Barker *et al* (1992; 1995) observed that *L. pneumophila* cells growing within phagocytes became more resistant to biocides and antibiotics. They were also found to be more invasive for mammalian cells, and more virulent and infective in studies using mouse models (Brieland *et al*, 1997; Cirillo *et al*, 1999). *L. pneumophila* has been shown to have two distinct phases of growth, which as mentioned, dramatically influence the phenotype of *L. pneumophila*. These are described as the 'replicative' and the 'active infective phase' (Rowbotham, 1986).

During replication, *L. pneumophila* cells are sodium resistant and non-motile. They do not express the *flaA* gene or produce flagella (Byrne and Swanson, 1998). Following replication however, *L. pneumophila* acquire numerous virulence traits, clearly adapted to the escape of

nutrient depleted host cells and dissemination to new cells. At this stage, the cells become short, highly motile rods. Byrne and Swanson (1998) observed numerous changes in the phenotype of postexponential phase cultures of *L. pneumophila*, which are directly related to an increase in virulence. These included the development of motility, sodium-sensitivity, cytotoxicity, osmotic resistance, and the ability to evade phagosome-lysosome fusion. This conversion to a virulent form appears to be modulated by the detection of a depleted amino acid supply. Therefore, when the bacteria detect a lowering level of available nutrients, they begin to express the virulence traits necessary for escape and dissemination to a new host cell. If cells are inhaled into a human lung during this highly virulent phase, Legionnaires' disease may ensue unless a strong and healthy host immune response is initiated (Byrne and Swanson, 1998).

Following this phase of high virulence, the cells may also enter a "stationary" phase, which is triggered by the 'stringent response pathway', and is regulated by the stationary-phase σ factor RpoS (Hammer and Swanson, 1999).

This is a mechanism used by cells to promote the long term survival of the organism, in environmental conditions which are usually unfavourable and nutrient deprived. The mechanism employed by *Legionella* is believed to be similar to that of *E. coli*, whereby this phase is characterised by a rapid decline in growth and protein expression and synthesis (Nash *et al*, 1984).

The figure below **(figure 1.5)** is a schematic summary of the different growth phases characteristic of the *L. pneumophila* life cycle. It also depicts some of the different phenotypes displayed by *Legionella* during each of the respective stages of growth.

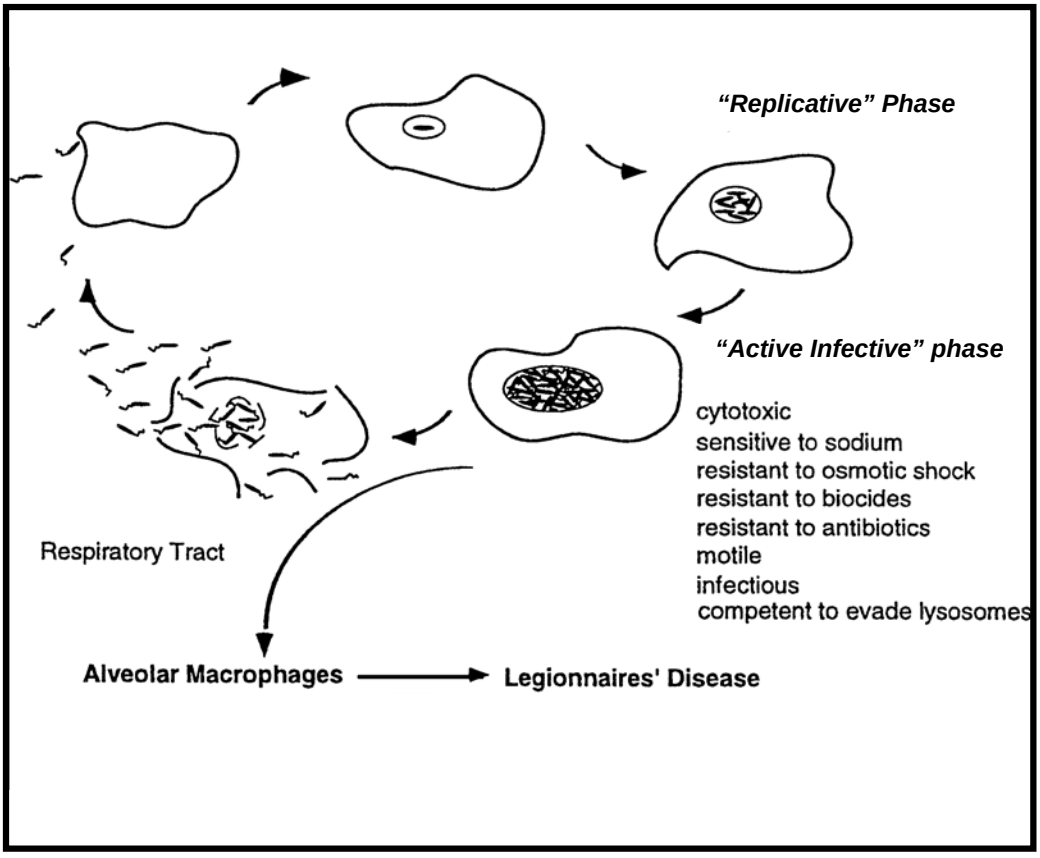


Figure 1.5 - Schematic representation of the life cycle and growth phases of *Legionella pneumophila*. The virulence traits expressed during the active infective phase are outlined (Based on figure from Swanson and Hammer, 2000).

1.3 Epidemiology

L. pneumophila remains an important cause of morbidity and mortality throughout the world. Following its identification in 1976, numerous outbreaks of Legionnaires' disease have occurred globally, many of which have resulted in significant numbers of human deaths. Of these, undoubtedly the worst outbreak to occur took place in 1999, at the Westfriese Flora Show in the Netherlands. Here, 231 people became infected with *L. pneumophila,* and 21 of these subsequently died (Wijgergangs, 1999).

In Australia, the largest outbreak to date occurred at the Melbourne Aquarium, in 2000. Here, culture methods were used in combination with urinary antigen detection to confirm the 119 cases of Legionnaires' disease, of which 4 patients died. Water samples taken from the cooling towers of the aquarium resulted in the isolation of *L. pneumophila* serogroup 1. The average age of patients in this outbreak was 63 years, and 57% of these were males. In Australia, the outbreak led to regulation changes for the industry. These included the compulsory registration of all cooling towers, new and old, mandatory risk management programs, annual audits, random inspections, including checks of maintenance and equipment records, and the introduction of an enhanced technical advisory and outbreak investigation service within the Department of Human Services (http://hnb.dhs.vic.gov.au).

Although Legionnaires' disease outbreaks are often widely publicised, the majority of cases are actually sporadic, and diagnosis of the cause is rarely made. Of the 20,000 cases of Legionnaires' disease believed to occur annually in the U.S, only around 1000 of these are reported (Marston *et al*, 1994., Montalbano *et al*, 1996). Person to person transmission of *Legionella* has never been reported, and the disease is most often community-acquired, but may also occur nosocomially. Of all the community-acquired pneumonias requiring hospitalisation, legionellosis only accounts for between 2% - 15% of cases (Marston *et al*, 1994). Nosocomially acquired disease however, is often more striking and severe, and mortality rates can be as high as 50%, which is most likely due to the immunocompromised status of most hospital patients (Carratala *et al*, 1994).

Even though almost 50 species of *Legionella* have been identified to date, by far the most prevalent species implicated with disease is *L. pneumophila*, and in particular, *L. pneumophila* serogroup 1. Although the incidence of disease due to other serogroups or

species appears to be on the increase, research into the pathogenic mechanisms of *Legionella* spp. is predominantly based on this species.

It has been well established that amoebae are the natural environmental reservoirs for *L. pneumophila*, and the organism is readily able to infect over 13 species of amoebae and two species of ciliated protozoa (Fields, 1996). The life cycle of *L. pneumophila* within protozoa is remarkably similar to that of its life cycle within macrophages (Figure 1.5), and many of the same virulence factors are required for infection, including the Macrophage invasion potentiator (Mip), and the protein products of the *dot/icm* genes (Prescott, 2005).

Amoebae are believed to be the perfect host for *Legionella* spp., as they are also ubiquitously present in the environment, including water sources, such as potable water supplies, and even heated reservoirs. A connection has also been made between the presence of both *L. pneumophila* and protozoan phagocytes in supplies of water linked with legionellosis outbreaks (Barbaree *et al*, 1986). In the environment, amoebae serve an important survival role for *Legionella*, protecting the intracellular organism from biocides and other harsh environmental conditions. Barker *et al* (1992; 1995) found that *Legionella* grown within phagocytes are more resistant to both chemical biocides, such as agents used for water sanitising, and antibiotics.

It has also been observed that *Legionella* organisms growing within amoebae are often highly virulent. For example, it has been observed that *Legionella* cells that are released from lysed amoebae are highly motile, which is a virulence trait also seen with cells grown within macrophages, and one which is associated with transmission (Byrne and Swanson, 1998; Rowbotham, 1986). In addition to this, it was observed by the group of Cirillo *et al* (1999; 1994) that compared to *Legionella* growing in broth, *Legionella* cells obtained from *Acanthamoeba castellanii* were able to enter human monocytes and a monocytic cell line with greater ease. They were also able to do this in a complement-independent manner.

1.4 Prevention

Legionella is a pathogen found world-wide, that is capable of causing a severe and life threatening form of pneumonia. Preventative measures are imperative in reducing the incidence of sporadic cases, or outbreaks of legionellosis. Sadly, many cases of Legionnaires' disease could have been avoided by the simple maintenance and control

measures now outlined by regulatory authorities, such as the Environmental Protection Agency (EPA), and the Center for Disease Control (CDC).

1.4.1 Control

The Center for Disease Control (CDC) recently altered their guidelines for the control and prevention of *Legionella* spp. in water systems. These guidelines were released in 2003, and are entitled the "Guidelines for Environmental Infection Control", and the "Guidelines for preventing healthcare-associated pneumonia" (http://www.cdc.gov).

Their previous guidelines were based on the premise that hospitals should only implement preventative measures once a case of Legionnaires' disease was identified. Similarly, environmental sources were only to be tested following the event of an outbreak (Freije, 2004). This is obviously not the right approach, and contradicts all forms of preventative measures. The guidelines were consequently updated in 2003, and now have a *proactive*, rather than a *reactive* approach. The new guidelines include laboratory testing of suspected Legionnaires' disease patients. The recommendation to laboratories is that both the culture of respiratory specimens, as well as the urinary antigen test, is implemented for diagnosis. They also added that it is important to review the availability and clinicians use of the diagnostic tests for Legionnaires' disease. Another addition to the guidelines is the recommendation to educate physicians and health care workers on prevention and control measures of *Legionella* and Legionnaires' disease. Lastly, the CDC recommends the periodic testing of potable water for *Legionella* in health care facilities that have patients who may be highly susceptible to legionnaire's disease, such as the immunocompromised.

The Environment Protection Agency (EPA) regulations appear to be somewhat more stringent in regards to preventative measures. Their guidelines entitled "Guidance Manual for the compliance with the filtration and disinfection requirements for public water systems using surface water sources" which is part of the "Institutional control of *Legionella*", recommends that hospitals, hotels and other large buildings have their water tested quarterly, throughout the year (Environment Protection Agency, 1991).

Disinfection of water can be performed by three different methods. These include chemical, physical, and thermal disinfection. Chemical disinfection of water sources which may pose a potential hazard for the spread of *Legionella*, such as air conditioning systems and cooling towers, is the main method employed to ensure that numbers of *Legionella* spp. organisms remain under control, and within permitted guidelines. An example of a physical method of water disinfection is membrane filtration. This method, however, is rarely used. Thermal disinfection is another method which is used quite commonly, and involves superheating of water to high temperatures (Best *et al,* 1984).

1.4.2 Disinfection measures

1.4.2.1 Chemical disinfection

In terms of chemical disinfection, there are numerous classes of disinfectants in use. These include the use of metal ions, such as copper and silver, and oxidizing or non-oxidising agents.

Oxidising agents include chlorine, bromine, iodine, chlorine dioxide, chloramines, halogenated hydantoins, ozone, and hydrogen peroxide.

Examples of non-oxidising agents used include heterocyclic ketones, guanidines, thiocarbamates, aldehydes, amines, thiocyanates, organo-tin compounds, halogenated amides, halogenated glycols and UV light. Kim *et al* (2002) recently reviewed the efficacy of these methods. They found that in general, the oxidising agents were more effective than the non-oxidising agents, with chlorine being a common and effective choice. Of the non-oxidising chemicals, they found that 2,2-dibromo-3-nitropropionamide (DBNPA) was the most effective, followed by glutaraldehyde.

Chlorine is in widespread use for the disinfection of water systems. Interestingly, *Legionella* spp. are much more resistant to chlorine than *E. coli* and other coliforms (Kuchta *et al*, 1993), and in order to control *Legionella* spp., concentrations of between 2–6 mg/L of chlorine are constantly needed (Lin *et al*, 1998). If the *Legionella* spp. are associated with amoebae, however, the minimal concentration required for disinfection increases to at least 4 mg/L (Kuchta *et al*, 1993).

The efficacy of chlorine was also found to be greater when used in water which was maintained at the higher temperature of 43°C, as opposed to 25°C. The disadvantage, however, is that chlorine decays faster at a higher temperature (Muraca *et al*, 1987). Another method which can be employed is termed shock hyperchlorination. This involves the periodic use of very high levels of chlorine (20–50 mg/L). This is followed by the replacement of the water in the system after 1–2 h with fresh water, and then maintaining the system with a low concentration of around 1 mg/L of chlorine (Lin *et al*, 1998). Although the use of chlorine may seem to be a relatively safe, cheap and effective means of disinfection, there are also several disadvantages associated with its use. Firstly, it may not be totally effective at eradicating *Legionella*, especially if they are associated with amoebae, or are present in biofilms. A second issue is the fact that chlorine is corrosive to pipes and construction materials. The price of replacing or maintaining the water system may therefore become very costly. A final concern is the health related risk to the development of chlorination by-products, such as chlorinated organics, particularly in systems used for domestic drinking water. This may also become problematic to waste water treatment plants, where the chlorine residue could prove toxic to the microorganisms used to process the waste water, rendering the system less effective.

1.4.2.2 Thermal disinfection

Kim *et al* (2002) also tested the efficiency of thermal disinfection, and found that this was effective for killing microorganisms at temperatures above >60°C (140°F).

In support of this finding, Rogers *et al* (1994) found that *Legionella* were able to survive in water at temperatures between 20 and 50°C, but were not recovered from water heated to 60°C. Similarly, Lin *et al* (1998) performed a study to determine the time required for a 90% reduction (ie. 1-log) of *Legionella* spp., at temperatures of 45°C, 50°C, 60°C and 70°C (113°F, 122°F, 140°F, and 158°F). They found that the times required for a 90% reduction, in minutes, were 2500, 380, <5 and <1 min, respectively.

According to Best *et al* (1984), effective thermal disinfection of hot-water distribution systems requires flushing of all outlets, faucets, and shower heads for at least 30 min at >60°C. Maintaining the temperature of the water at 60°C following this treatment is also

known to be effective at preventing the re-establishment of the organism (Furuhata *et al*, 1994).

Vickers *et al* (1987) performed a study of *L. pneumophila* in hot water systems, and found that vertical storage tanks were contaminated with the organism more commonly than horizontal tanks. They also found that older tanks were contaminated more often than newer tanks.

Overall, thermal disinfection is an effective means of eliminating *Legionella* from water systems. It is relatively inexpensive, and does not corrode pipes or construction materials as do some of the chemical methods. One disadvantage however, is the risk to the operator of scalding, due to the high temperature, and large volume of water involved. However, if caution is exercised, it remains an affordable and effective option for prevention of *Legionella* growth and dissemination.

1.4.3 Vaccination

Prevention of *Legionella* is unfortunately, not yet possible by means of vaccination. Various groups have analysed potential vaccine candidates, but as yet, none have satisfactorily made their way to the commercial market (Mims *et al*, 2004).

Various groups began to analyse the potential of different *Legionella* spp. antigens for use as vaccine targets, as early as the late 1980's. One of the earlier groups was Blander and Horwitz (1989). They investigated the protective potential of the Major secretory protein (Msp) of *L. pneumophila*, using a guinea pig model. Interestingly, they found that in the guinea pig model, the Msp of *L. pneumophila* was able to elicit both a humoral and cell-mediated immune response. It was able to protect the guinea pigs from an aerosol challenge with a high dose of virulent *L. pneumophila*. The group found that with a 40 μg dose, injected twice over a three week period, the guinea pigs experienced only limited multiplication of *L. pneumophila* in their lungs following the aerosol challenge.

Similarly, Blander *et al* (1989) analysed the potential of a live avirulent mutant of *L. pneumophila* to elicit a protective immune response in guinea pigs. They found that the mutant did not cause disease in the guinea pigs, and it did not revert to a virulent form with passage through the guinea pigs.

They observed that the immunised guinea pigs developed both a strong humoral and cell mediated immune response to wild-type *L. pneumophila*, and were protected against an aerosolised challenge with wild-type *L. pneumophila*. The group therefore concluded that the study demonstrated the potential for use of this attenuated *L. pneumophila* mutant, and that it may prove useful in the vaccination of people in the high risk group for developing Legionnaires' disease, such as cigarette smokers, and patients on immunosuppressive therapy.

Belyi *et al* (1996) determined that a vaccine produced from live *Francisella tularensis,* an organism genetically related to *L. pneumophila,* was able to protect guinea pigs which had been immunised, from a lethal aerosol challenge of virulent *L. pneumophila*. The group observed that immunisation with the tularemia vaccine protected over 80% of the challenged guinea pigs. The group tried to determine which of the bacterial components were responsible for eliciting the protection by isolating cell wall components of the organism. Unfortunately the individual cell wall components failed to induce the same protective response, and the components responsible for eliciting the response remain unidentified.

Recently, Ricci *et al* (2005) analysed the protective potential of the *L. pneumophila* flagella, using the A/J mouse model. The group immunised the mice with purified flagellum protein, and found that upon challenge with virulent *L. pneumophila*, the mice displayed a 100% survival rate, due to the elicitation of a strong innate and adaptive cell-mediated immune response. This protective response was found to last for around thirty days, and protected mice from challenges with different serogroups of *L. pneumophila*.

The group concluded that the flagellum protein is highly immunogenic, and is capable of protecting A/J mice against a lethal *L. pneumophila* challenge, due to the stimulation of both natural and acquired T-cell-mediated immune responses.

The search for an ideal vaccine candidate for *Legionella* continues, and remains to be ascertained. If readily available and inexpensive, the vaccine would be a useful prophylactic tool for the elderly and immunocompromised. Due to the comparatively small target group, and the relative infrequency of legionellosis cases, it may be however, that there is insufficient demand and financial support for the development of a *Legionella* vaccine. As with all cases of vaccine development, the process can often take numerous years of laboratory trials, testing, and monetary input before the vaccine is ready for the commercial market.

1.5 *Legionella* Outer Membrane Proteins/Components

1.5.1 *M*acrophage *I*nvasion *P*otentiator (Mip)

The **M**acrophage **I**nvasion **P**otentiator (Mip) family of proteins are a group of surface bound proteins which display peptidylprolyl *cis:trans* isomerase (PPIase) activity. They are an effective virulence attribute possessed by a few intracellular organisms, which include *L. pneumophila*, (which was the first organism to have the Mip recognised as an important virulence factor), *Neisseria gonorrhoeae*, *Chlamydia, E. coli* and *Tryponosoma* (Leuzzi *et al*, 2005).

Bacterial Mips are believed to be the prokaryotic homolog to the human FK506-binding protein (FKBP) family of enzymes. FK506-binding proteins, also known as Immunophilins, bind to immunosuppressive drugs, such as the macrolactones FK506 and rapamycin (Bell *et al*, 2006).

It is now quite well known that Mips are a critical factor in the successful survival of intracellular organisms, as they play a very important role in the intracellular infection of a key member of the immune system, the human professional phagocyte, or macrophage. There is now ample evidence which suggests that deletion or mutation of the *mip* gene severely reduces the virulence level of the bacteria (Cianciotto *et al*, 1989; Cianciotto and Fields, 1992; O' Connell *et al*, 1995). Recently, the group of Debroy *et al*, (2006) identified the target molecule of the Mip protein as a phospholipase C, although more work is needed to determine if this new information explains the role of the Mip in pathogenesis.

1.5.1.1 The Mip protein within the species *L. pneumophila*

A considerable amount of attention has been focused on the Mip protein of *Legionella*, particularly of *L. pneumophila*. This is most likely due to the fact that *L. pneumophila* is a ubiquitous pathogen which has the potential to cause life threatening illness, and of which the Mip has been shown to be an imperative part of its pathogenesis.

The Mip protein of *L. pneumophila* is a surface exposed protein, that has been shown to contribute to *L. pneumophila* infection of both protozoan and human macrophage cells (Cianciotto *et al*, 1992). The DNA sequence of the *L. pneumophila mip* gene was elucidated by the group of Engleberg *et al* (1989). It was found to encode a 24 kDa protein, which included a secretory signal sequence, and was believed to be transcribed monocistronically.

The crystal structure of the *L. pneumophila* Mip protein was recently determined by Riboldi-Tunnicliffe *et al* (2001). They found that the protein contains quite an atypical and unusual structure. It was found to be homodimeric, as opposed to the majority of other FKBPs, which are monomers **(Figure 1.6)**. It was also found to have a unique N-terminal sequence that is significantly different to that of the Mips of other organisms, and interestingly, was found to have a C-terminal domain which shares around 35% sequence similarity with the human protein FK506-binding protein (FKBP12).

Each monomer is described as having a 'dumbbell' shape due to its two structurally distinct domains being linked by a long α-helix. These domains consist of two anti-parallel α-helices, joined by a loop of six residues. It is these two α-helices which form the main biologically active dimer of the Mip protein. An eight-residue loop then connects the N-terminal domain to a linker helix. This linker helix, designated the α3 helix, contributes greatly to the uniqueness of the Mip protein. At a length of 45 residues, this helix is believed to be the longest free-standing α-helix of any protein. It is not stabilised by any external bonding to other parts of the protein, but instead, is stabilised internally by hydrogen bonds and salt bridges (Riboldi-Tunnicliffe *et al*, 2001).

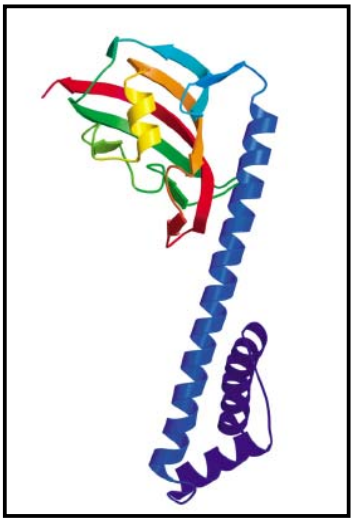


Figure 1.6 – Schematic depiction of the crystal structure of the Mip monomer. The coils represent α-helices, whilst the arrows correspond to β-strands. The N-terminal region is colored blue, and the C-terminus is red (Riboldi-Tunnicliffe *et al*, 2001).

1.5.1.2 Role of the Mip in *L. pneumophila* pathogenicity

Functional investigation of the *mip* gene through analysis of the effects of mutations has been performed by several groups (Cianciotto *et al*, 1989; Cianciotto *et al*, 1990; Cianciotto *et al*, 1992; Hurley *et al*, 1993; Cianciotto *et al*, 1995; Shi *et al*, 2006).

It was observed that a site-specific mutation introduced into the *mip* gene resulted in *L. pneumophila* having a significantly reduced ability to infect human alveolar macrophages, as well as the human macrophage-like cell line U937 (Cianciotto *et al*, 1989).

When the growth curves of *mip*-mutants were compared to the parent strain in U-937 monolayers, it was noted that there were significant differences between the two. Even though equal numbers of bacteria were used to infect the monolayers, there was found to be a reduction of around 10-fold in the number of *mip*-mutants recovered after 40 hours of infection. This number dropped to around 100 fold fewer mutants recovered after 3 days post-inoculation.

However, the group found that once inside the U-937 cells, the mutant organisms were able to grow at a comparable rate to the parent strain. They therefore explained this apparent

difference in growth curve kinetics by the fact that the mutant cells are probably unable to effectively initiate successive rounds of replication within the U-937 monolayer. They were then able to demonstrate that this was most likely the case by infecting the monolayer with 10 fold more *mip*-mutants, so that an equivalent number of organisms were present at the 40 hour post-infection point. When the growth curve was again analysed, it was observed that the rates of growth for both the parent strain and the mutant strain were indeed similar.
This observation may be strengthened by the findings of Wieland *et al* (2002) who studied the regulation of the *mip* gene, and found that its expression was repressed a short time after the infection of a cell. Therefore, Mip expression is probably only important during the initial stages of infection.

1.5.1.3 Distribution of the Mip gene within the genus *Legionella*

Distribution studies have also been performed on species of *Legionella* other than *L. pneumophila*. Cianciotto *et al* (1990) performed Southern hybridisation and immunoblot analyses on numerous *Legionella* strains and serogroups, in order to determine the presence and expression of the *mip* gene, and Mip protein product. They found that 14 serogroups of *L. pneumophila* encoded a *mip* gene, and expressed a 24 kDa gene product. Reactivity of all 14 serogroups tested was observed when immunoblotted using anti-Mip monoclonal antibodies. However, when Southern hybridisation reactions with *mip* DNA probes were performed on 29 other species of *Legionella*, they found that reactivity was only observed when the stringency conditions of the reaction were reduced. This indicated that the gene was present, but that there were variations in parts of the nucleotide sequence. Immunoblotting of whole cell lysates of *Legionella* species performed with anti-Mip antibodies also revealed that these strains all expressed a Mip protein product, ranging between 24-31 kDa in size (Cianciotto *et al*, 1990).
Interestingly, when hybridisation reactions were performed on *Legionella* strains with DNA probes designed from the 5' end of the *mip* gene, reactivity was stronger than with probes designed from the 3' end. This indicated that the gene appeared more conserved between species at the 5' end, and that more sequence variability exists at the 3' end.
Cianciotto *et al* (1990) also performed Southern hybridisation reactions with *mip*-based DNA probes using bacteria outside the genus *Legionella.* Under the conditions used by the

group, they found that there was no reactivity with any of the other bacterial species tested. These included *E. coli*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Mycoplasma pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Streptococcus pneumoniae*. They concluded that all of the *Legionella* strains tested expressed a Mip-like protein, and that all of the *Legionella pneumophila* serogroups tested possessed a *mip* gene very closely related to that of *Legionella pneumophila* serogroup 1.

O'Connell *et al* (1995) analysed the *mip* gene of *Legionella micdadei*, another important and relatively common cause of Legionnaires' Disease, which has also been implicated in abscess formation and cellulitis (Halberstam *et al*, 1992; Kilborn *et al*, 1992). The group mutated the *mip* gene by allelic exchange, which resulted in the complete loss of Mip protein expression. They then performed infectivity assays using the U-937 cell line, as well as the amoebal parasite *Hartmanella vermiformis*. The results showed that similarly to *L. pneumophila*, the Mip of *L. micdadei* is also important for intracellular infection of the organism, and that mutation of the gene significantly impairs its ability to infect macrophages. They also found that the *mip* mutant had a reduced ability to survive within the amoebae, indicating that the protein may play a role in resistance to killing by the host cell. Overall it was shown that the Mip of *L. micdadei* shared a very similar function to that of the *L. pneumophila* Mip.

1.5.1.4 Mip-Based Detection Systems

The *mip* gene has been used as the target for a genotypic classification scheme for the genus *Legionella* (Ratcliff *et al*, 1998). It was found to be able to clearly distinguish between species of *Legionella*, and showed more than twice the discriminatory ability of the commonly used 16S rRNA gene. In addition to the nucleotide variation which occurs between species throughout the *mip* gene, the fundamental feature of the scheme relies on a hypervariable region of DNA adjacent to the proteins' signal sequence coding region.

The classification scheme involves the amplification and subsequent DNA sequencing, of around 700 nucleotide bases of DNA (between 661 to 715 bp, depending on the *Legionella* species). The study found that the *mip* gene was quite stable i.e. that there was no evidence of homologous recombination, and that the classification scheme correctly identified 39 of

40 *Legionella* species. It was also successfully able to further group 26 serogroups or reference strains for these *Legionella* species.

Numerous research groups have developed methods for detecting *Legionella* spp. by the sensitive and specific technique of PCR (Bej *et al*, 1991; Kessler *et al*, 1993; Koide and Saito, 1995; Murdoch *et al*, 1996; Wellinghausen *et al*, 2001). More recently, a real-time PCR assay was developed for the detection of *L. pneumophila* in clinical samples, based on the *mip* gene (Wilson *et al*, 2003). It was shown that the *mip* gene served as a reliable and effective target gene for the identification of *L. pneumophila* in the clinical samples tested. However, they noted that two species of *Legionella*, namely *L. worsleiensis* and *L. fairfieldensis* could possibly be falsely identified as *L. pneumophila*, since a BLAST sequence search performed by the group revealed a highly similar sequence match for the *mip* gene, in the regions targeted by the assay. However they could not support this hypothesis because they did not have access to these strains.

1.5.2 Lipopolysaccharide (LPS)

Lipopolysaccharide (LPS), has for numerous years now, been considered a contributor to *Legionella* pathogenesis. It has been linked to intracellular growth, virulence and serum resistance, and is considered to be the major immunodominant antigen of *L. pneumophila* (Luneberg *et al*, 1998).
LPS is a complex molecule, which is present in the outer membrane of gram negative organisms, and is often responsible for the elicitation of innate immune responses from infected hosts (Girard *et al*, 2003).

The structure of the LPS of *L. pneumophila* differs significantly from the LPS of other gram negative organisms, in that its lipid A component is composed of specific long chain fatty acids, which are believed to be the rationale for the molecules' relatively low endotoxic properties (Wong *et al*, 1979). The O-chain of the molecule, termed 'Legionaminic acid', is composed of a homopolymer of the unique sugar, 5-acetamidino-7-acetamido-8-*O*-acetyl-3,5,7,9-tetradeoxy-L-*glycero*-D-*galacto*-nonulosonic acid. It is this sugar which is responsible for the high hydrophobicity of the cell surface, and may therefore play a role in

bacterial adherence to alveolar macrophages, or amoebae (Zähringer *et al*, 1995). Helbig *et al* (1995) postulated that an epitope adjacent to the 8-O-acteyl group of the Legionaminic acid is involved in the virulence of *L. pneumophila*.

The inner core oligosaccharide of the molecule does not contain heptose sugar molecules, but is composed of 2-keto-3-deoxy-D-*manno*-oct-2-ulosonic acids, which is similar to those of other enterobacterial core oligosaccharides (Neumeister *et al*, 1998). The outer core of the molecule is also hydrophobic due to *N*- and *O*-acetyl groups, in addition to 6-deoxy sugars **(see Figure 1.7)**.

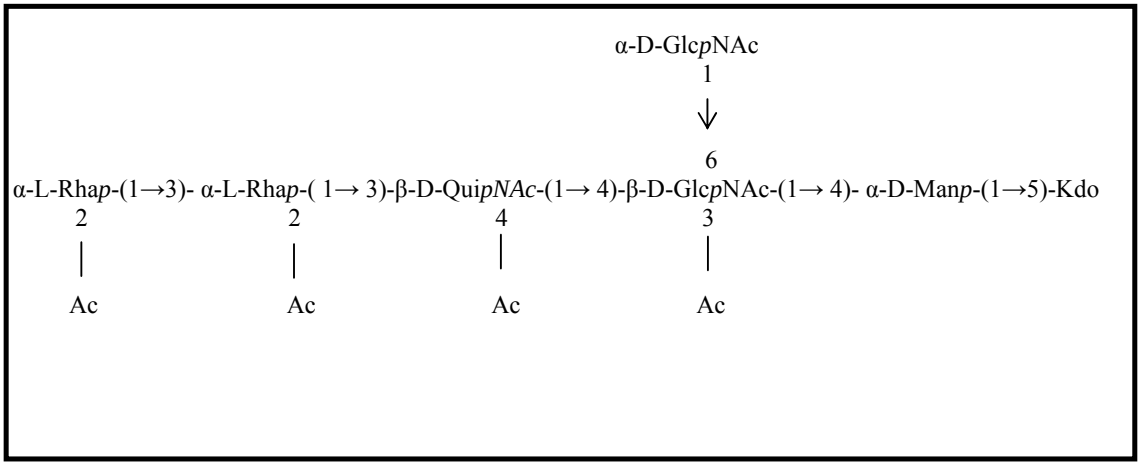


Figure 1.7 - The highly *O*-acetylated core heptasaccharide of *Legionella pneumophila* LPS, where Kdo is 3-deoxy-D-*manno*-octulosonic acid and QuiNAc is 2-acetamido-2,6-dideoxyglucose (Knirel *et al*, 1996).

1.5.2.1 LPS - its role in typing and identification of *Legionella*

Ciesielski *et al* (1986) performed a study to characterise the LPS of *L. pneumophila*, and to establish the relationship between LPS and the specificity of serogroups. They subjected the LPS from all *L. pneumophila* serogroups tested (serogroups 1-6) to SDS-PAGE analysis, and found that the migration pattern of these LPS samples were very distinct, and significantly different to that of other gram negative organisms. Each of the serogroups were seen to be associated with multiple complexes and have a smooth LPS profile. This smooth type LPS

correlates well with the work of Horwitz and Silverstein (1981), who determined that these strains of *L. pneumophila* displayed serum resistance. In order to establish if LPS was a determinant in serogroup specificity of strains, the group also performed immunoblots with proteinase K–treated whole cell lysates of *L. pneumophila* serogroups 1-6, using monkey immune serum which was serogroup specific. They found that only homologous antisera reacted with the lysates, indicating that the LPS was a determinant for the specificity of each serogroup. Although there was some very slight cross-reactivity between a few low molecular weight antigens of serogroup 6 with immune serum 5, generally the antiserum would only be reactive against its homologous serogroup, and not the other five.

The LPS of *Legionella* is an antigenic structure which is often used for typing and identification of the organism. Jurgens and Fehrenbach (1995; 1997) have been studying the LPS of *Legionella* for several years now. In 1995, the group published work in which they analysed the cross-reactivity of *L. pneumophila* serogroups 1-14. When the group separated the LPS of serogroups 1-14 by SDS-PAGE, they found that most strains exhibited tight ladder like migration patterns. This was not the case however, with serogroups 4, 5, 6, 12 and 13, whereby bands were quite widely separated. They found that overall, the banding patterns between serogroups 1-14, as well as the species *L. bozemanni, L. gormanii* and *L. micdadei* were significantly different from each other, and quite unique. The only similarity observed between banding patterns was amongst strains of a particular serogroup. In this case, the group tested 8 strains of *L. pneumophila* serogroup 1, and found them to be highly similar. In contrast to the group of Ciesielski, (1986) who tested cross-reactivity between serogroups 1-6, and found some cross-reactivity with serogroup 5 against the LPS of serogroup 6, Jurgens and Fehrenbach (1997) found cross-reactivity with serogroup 5 immune serum, against the LPS of all serogroups 1-14. It did not react however, with any other *Legionella* species, or other gram negative bacteria. From this, the group concluded that polyclonal antiserum raised against *L. pneumophila* serogroup 5, would be useful in the detection of all *L. pneumophila* serogroups. They also found that when separated by SDS-PAGE, the LPS of *L. pneumophila* serogroups 1-14 is quite unique and can therefore serve to distinguish and type the different serogroups of *Legionella pneumophila*. The technique however, was not useful for the further differentiation of the serotypes into subtypes, as their LPS profiles were too similar.

Jurgens and Fehrenbach (1997) also recognised the importance and success of using LPS to type other bacterial species, and therefore considered that this concept may also be applicable to *Legionella*. They therefore separated LPS preparations of numerous *Legionella* species by SDS-PAGE, and analysed results to determine whether the LPS pattern of 28 ATCC reference strains matched those of 430 wild-type isolates. They found that generally, the LPS pattern of the wild-type strains corresponded to their respective reference strain. However, they did observe some slight variations with some strains, in the form of either an additional or absent band. They concluded that this discrepancy in results may in fact have been as a result of different LPS concentrations in samples, and not necessarily variations in the LPS structure of the strains. The group did mention however, that the strains required further testing, but that the method showed potential due to both the stability of the LPS molecule and the relative ease of the test.

Further to the concept of using LPS to differentiate between strains of *Legionella pneumophila*, Helbig *et al* (1997) performed a study whereby they created a panel of 98 monoclonal antibodies, using reference strain serogroups. Each serogroup of *L. pneumophila* was said to possess at least one epitope which was specific to that serogroup only, allowing the creation of the monoclonal antibodies which specifically target a particular serogroup. The panel was designated the 'Dresden *Legionella* LPS MAb panel". A total of 165 clinical and 899 environmental isolates were included in the testing, and the group found that overall, the method of distinguishing serogroups by their LPS epitope patterns was effective. A major difficulty encountered during the development of the monoclonal antibodies however, was the fact that the antibodies developed against some serogroups (serogroups 4, 5, 8, 10, 12 and 14) shared epitopes which were cross-reactive with several other serogroups. Serogroups 4 and 5 were particularly problematic, as no serogroup specific epitopes for these strains were found, and a two-monoclonal antibody system had to be implemented for their identification. Overall, Helbig *et al* (1997) concluded that the monoclonal antibody system was valuable, and recommended its use in routine and clinical laboratory testing.

1.5.2.2 LPS, Macrophages and the immune system

The LPS of *Legionella*, as with other gram negative bacteria, can trigger a cascade of immunological activity within a host. It is known to activate macrophages, but can lead to

the development of septic shock in a host, due to the release of cytokines, such as Interleukin-1α (IL-1α), IL-1β, IL-6 and tumour necrosis factor α (TNF-α). Although endotoxic shock can result from excess secretion of cytokines such as TNF-α, the release of these cytokines also protects host cells against further bacterial infection.

Arata *et al* (1993) studied the effect of *L. pneumophila* LPS-activated macrophages, and the resulting non-permissiveness of these macrophages to further infection by intracellular bacteria. The group found that when LPS of *L. pneumophila* was injected into the peritoneal cavity of A/J mice, the macrophages became activated and were then resistant to subsequent infection with *L. pneumophila in vitro.* Interestingly, if a batch of normal, non-activated macrophages was co-cultured with the treated, LPS-activated macrophages, the entire population of cells became non-permissive to further intracellular infection. The group examined whether this effect was due to the release of cytokines by the activated macrophages, such as IL-1, IL-6 or TNF-α, but was surprised to find that the levels of these soluble factors in the cells were not notably high. The group also tested the theory that the transition of macrophages from permissiveness to non-permissiveness may have been due to free LPS released by cells following injections of the endotoxin. They therefore pre-treated cells with Polymyxin B, a known inhibitor of LPS activity. Again, they found that this did not significantly alter the ability of LPS-activated macrophages to induce non-permissiveness in the unactivated macrophages, therefore indicating that it was most likely not LPS released from the activated macrophages that was eliciting this effect. However, the group noted that free or macrophage–bound LPS could not be totally ruled out as a cause of this phenomenon. Although the mechanisms involved still remain vague, the group postulated that there may be some kind of LPS-binding protein involved, which is able to bind to LPS, and present this LPS-LPS-binding protein complex to naive macrophages, enabling them to become activated and non-permissive, at much lower concentrations of LPS than normal. Following these initial experiments, several groups discovered that there are numerous molecules involved in the activation of macrophages and monocytes. One of these is the LPS-binding protein (LBP).

1.5.2.2.1 LPS Binding Protein (LBP)

Tobias *et al*, (1995) investigated the mechanism of LPS activation via the formation of a complex between the 60 kDa plasma glycoprotein known as the LPS binding protein (LBP), and the 55 kDa glycoprotein, CD14. This complexation process is believed to occur in a variety of mammalian cell types, including macrophages, neutrophils, endothelial cells, smooth muscle cells, and some epithelial cell lines (Haziot *et al*, 1988).

1.5.2.2.2 GPI-anchored cell-surface protein CD14

CD14, a cell surface protein, is found in macrophages, monocytes and neutrophils in the form of a glycerophosphoinositol tailed membrane protein (mCD14). It is believed that when LPS comes into contact with LBP, this initiates the binding of the LPS/LBP complex to mCD14. This in turn, initiates cellular activation (Wright *et al*, 1990). It was determined that inactivation of CD14 through the use of monoclonal antibodies, prevented the release of the important cytokine TNF-α. Such cytokines are critical in the activation process, as they prime leukocytes to respond to circulating LPS at very low amounts (ng/ml) (Wright *et al*, 1990).

1.5.2.2.3 Toll-Like Receptor 2 (TLR2)

Another recently elucidated mechanism of phagocyte activation is the involvement of the signal-transducing molecule, Toll-like receptor 2 (TLR2). It was previously believed that *Legionella* used the molecule TLR4 (Poltorak *et al*, 1998) as a signal transducer. However, the group of Girard *et al* (2003) recently carried out some follow up work that they had been performing on the LPS of *Rhizobium* where they had found that CD14 expression in bone marrow cells (BMCs) was occurring by a TLR4-independant mechanism. This was believed to be due to the lipid A region of the *Rhizobium* LPS, which structurally contains a very long fatty acid chain. The LPS of *Legionella* contains a similarly long fatty acid chain, and was

therefore included in the investigation. Interestingly, the group found that *Legionella* could also activate BMC's by TLR2, instead of TLR4. It is believed that the long chain fatty acids are not only responsible for the use of TLR2 mediated responses, but that they also reduce the reactivity of the LPS. The group concluded this based on the fact that an almost 100-fold higher concentration of *Legionella* LPS is required to activate BMCs, than for other enterobacteria or *B. pertussis* LPS.

1.5.2.2.4 LPS Activation of macrophages – the 'oxidative burst'

The 'oxidative burst' is a term used to describe a mechanism employed by cells to kill intracellularly growing organisms. This is accomplished by the release of O_2^- and H_2O_2 oxygen metabolites which are bactericidal to organisms such as *L. pneumophila,* and are ultimately responsible for their detriment.

Kura *et al* (1994) investigated the role of the oxidative burst of macrophages in preventing infection by intracellular organisms, following activation by LPS. A murine macrophage-like cell line was used to observe permissiveness, as well as a mutant cell line, LPS1916, which was defective for the oxidative burst following activation by LPS. *L. pneumophila* serogroup 1 was infected into the monolayers, and the group then determined the extent of infection by performing CFU assays. The group found that under normal circumstances, both cell lines were permissive for infection by *Legionella pneumophila*. Pre-treatment of the cell lines with IFN-γ, which has the same effect as stimulation by LPS, caused the parent cell line to become non-permissive to multiplication by the *L. pneumophila* cells. However, the mutant cell line did not become resistant to further infection.

A theory which had been postulated by other groups was the supposition that death of intracellular organisms by human monocytes was more likely due to the ability of macrophages to starve intracellular organisms of iron acquisition by minimising their expression of transferrin receptors (Byrd *et al*, 1989, 1993). However Kura *et al* (1994) found that levels of iron were quite high in cells following LPS stimulation.

Kura *et al* (1994) also found that it was the oxidative burst, and not the nitrite burst which was the critical bactericidal mechanism used by activated macrophages. They demonstrated this by inhibiting the production of cellular nitrite, and observing the effect this modification

had on the ability of the activated monocytes to inhibit bacterial growth. They found that there was no significant difference, indicating that the nitrite burst was in fact not an imperative factor in the inhibition of intracellular growth by macrophages.

1.5.2.3 *Legionella* LPS Phase Variation

The involvement of *Legionella* LPS in the virulence and phase variation of the organism has been studied for numerous years. It appears, however, that the precise mechanism of this involvement is yet to be fully understood. Several groups have been involved in the progression of this understanding.

Edelstein *et al*, (1985) analysed the changes in virulence that occur in cells grown over a variety of temperatures. They developed monoclonal antibodies to the organisms, and then analysed the binding ability of the antibodies to cells when grown at different temperatures. The group found that changes occurred in the structure of the LPS of cells which no longer facilitated binding of the antibodies. The group realised, however, that there may be other surface molecules involved, which are also affected by the change in growth temperature.

The group of Lüneberg *et al*, (1998) investigated the role of *L. pneumophila* LPS in virulence of the organism. To achieve this, the group raised monoclonal antibodies against the LPS of *L. pneumophila* serogroup 1. By doing so, they were able to identify an *L. pneumophila* LPS mutant. By performing assays using the human macrophage-like cell line HL60, the group was able to determine that although the mutant was able to enter the host cells, it was not able to replicate intracellularly. In addition to this, the mutant strain rapidly succumbed to serum complement factors, whilst the parent strain was virtually serum resistant. From this, the group concluded that the ability of *L. pneumophila* to resist serum complement factors is most likely due to the LPS carbohydrate moiety of the organism. However the group also acknowledges that there may be other cell surface molecules involved, which may have been altered due to the mutation of the LPS.

In addition to this finding, was the remarkable discovery that when the LPS mutant strain of *L. pneumophila* was injected into guinea pigs, it displayed an unstable phenotype, and appeared to revert from the mutant form, which was unable to bind to the monoclonal antibodies, back to the wild-type, which was able to bind the antibodies. This switching of

phenotypes was believed to be most dramatic *in vivo*, in the guinea pig model. Finally, the group also found that when they recovered bacteria from the experimental animals, there was an increase in the percentage of wild-type cells (from 8% to 35%), indicating that there was selective pressure occurring in favour of the wild-type phenotype.

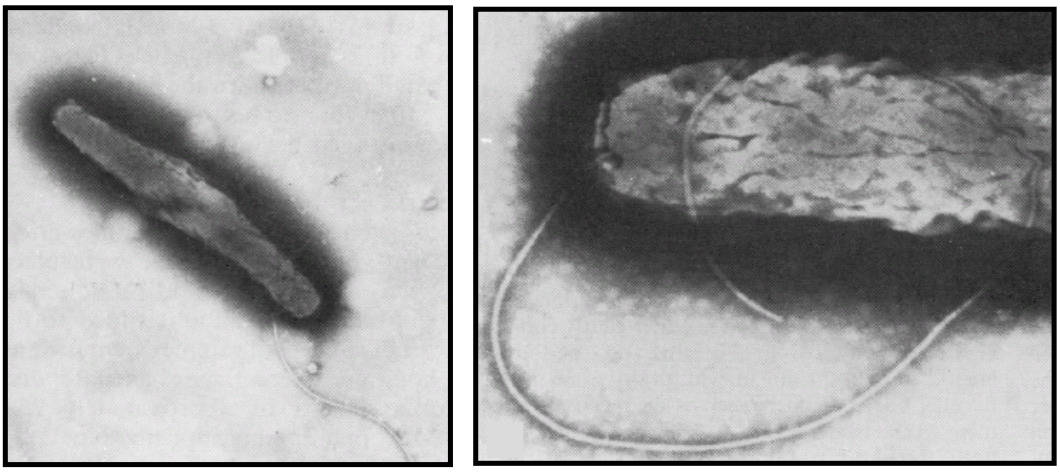
Several years after this work, the same group of Lüneberg *et al*, (2001) was able to elucidate the molecular mechanism behind the ability of *L. pneumophila* to vary their LPS by phase variation. The group discovered 30 genes encoded on a 30 kb unstable genetic element. They discovered that the organism is able to alter the cellular location of this element from a chromosomal location, when the cells are in a wild-type phenotype and virulent, to being present as a high copy plasmid in the cell, where they express a different LPS epitope pattern, and are no longer virulent. The group was therefore able to conclude that the phase variation expressed by *Legionella* is most likely defined by the continuous changing, by excision and insertion of this genetic element, from the chromosomal to plasmid locations.

1.5.3 Flagella

In many organisms, including *Legionella* spp., the flagellum is believed to be an important mechanism in bacterial pathogenesis. They provide organisms with motility, which is often a critical step in bacterial dissemination and spread (Salyers and Whitt, 2002).

The flagellum of *Legionella* spp. is known to consist of a 47 kDa filament subunit, and the organism has been characterised as having single or multiple, polar or subpolar flagella **(Figures 1.8a and 1.8b**, Rodgers *et al*, 1980; Elliot and Johnson, 1981). The work of Rowbotham described that flagellum expression and motility were associated with different growth phases of *Legionella* spp. (Rowbotham, 1986). These were described as the 'multiplicative phase', and the 'active infective phase'.

During the multiplicative phase, the bacteria are typically multiplying rapidly, and do not express flagella, and are hence non-motile. During the active infective phase, the bacteria become highly motile. During this stage, the organisms are typically in the later stages of infection, where they are lysing host cells, and disseminating. Motility during this stage is imperative for cells, as they are usually seeking to find new host cells to infect (Hammer and Swanson, 2000).



(a) (b)

Figure 1.8 - Electron microscopic image of flagella on *L. pneumophila*, negatively stained with 1% phosphotungstic acid, pH 6.5 (a) *L. pneumophila* serogroup 1 (Cambridge 1) from enriched blood agar medium, displaying a single subpolar flagellum, x 22,000. (b) *L. pneumophila* sgp 1 (Cambridge 2) from enriched blood agar medium displaying a single polar flagellum, x 66,000 (Taken from Rodgers *et al*, 1980).

Pruckler *et al*, (1995) investigated the relationship between flagellum expression and the intracellular growth of *Legionella*. They found that although the flagellum structure may not be essential for virulence, it may play an important role in the infection of amoebae, and the human macrophage-like cell line, U937. Furthermore, Dietrich *et al* (2001) reinforced this view by stating that although the flagellum was not necessarily involved in the replication of *Legionella*, it was highly likely to be implicated in *Legionella* invasion of host cells. In more recent years, a more in depth understanding has arisen of the role of the flagellum in *Legionella* pathogenesis. It is now universally recognised that flagella are involved in the transmission of the organism to new host cells, and that expression is upregulated during the stationary phase of growth in broth and whilst in the late-stage replication vacuoles of amoebae and macrophages (Heuner *et al*, 1999). If a mutation is introduced into the gene found to encode for the flagellum, designated *flaA,* the mutant organisms have a reduced ability to invade cells. Interestingly, following a cycle of intracellular replication, the *flaA* mutants also have difficulty lysing the host cell (Merriam *et al*, 1997).

1.5.4 Pili / Fimbriae

Pili, or fimbriae of gram negative bacteria are described as short, hair-like appendages that are much finer than flagella (3-10 nm diameter), and which are usually not involved in bacterial motility. Pili are usually involved in facilitating the attachment of organisms to solid surfaces or host tissues, and occasionally assist in the twitching motility observed in organisms such as *Ps. aeruginosa*, or *N. gonorrhoeae* (Prescott, 2005).

Legionella were found to possess pili by transmission electron microscopy studies, shortly after the discovery of the bacterium (**see Figure 1.9**, Rodgers *et al*, 1980). Since then, there has been a significant amount of research performed on the pili of *Legionella*, in order to fully comprehend its role in *Legionella* pathogenesis, with specific emphasis on its function in adherence of the organism to host cells.

Stone and Abu Kwaik (1999) investigated the genetic regulation of pilus expression in *L. pneumophila*. Interestingly, they found that *L. pneumophila* expressed pili of different lengths (**see Figure 1.10**, Stone and Abu Kwaik, 1999), which they believed was due to two sets of pilin encoding genes. By performing mutagenesis studies, the group was able to determine that the gene *pilEL* encoded for the longer type of pilus. This pilus was found to be related to the type IV pili of *Neisseria* spp. and *Pseudomonas* spp., and mutation of this gene resulted in a loss of these long pili on the surface of *L. pneumophila*. Without the expression of these long pili, the mutants were impaired in their ability to attach to cultured epithelial cells, macrophages, and protozoan cells.

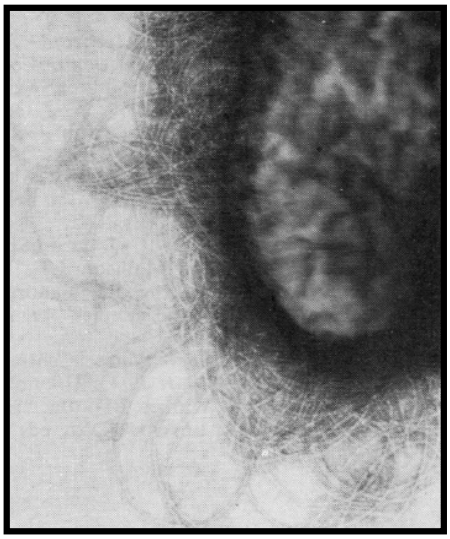


Figure 1.9 - Electron microscopic image of pili on *L. pneumophila* (Togus 1), negatively stained with 1% phosphotungstic acid, pH 6.5 displaying a mass of dense, curly pili, x 60,000 (Rodgers *et al*, 1980).

Liles *et al* (1998) was able to discover and analyse the gene locus responsible for pilus biogenesis, whilst they were in the process of analysing *L. pneumophila* iron acquisition genes. The genes were designated *pilB, pilC* and *pilD*, and were found to share significant similarity with the pilin biogenesis genes of *Ps. aeruginosa*.

By growing the *L. pneumophila* strains at the lower temperature of 30°C, the group observed that the expression of the operon was up-regulated, indicating that the piliation of *L. pneumophila* is temperature regulated. By performing Southern hybridisation reactions, the group was also able to determine that the pilin gene cluster appears to be conserved between the genus *Legionella* (Liles *et al,* 1998).

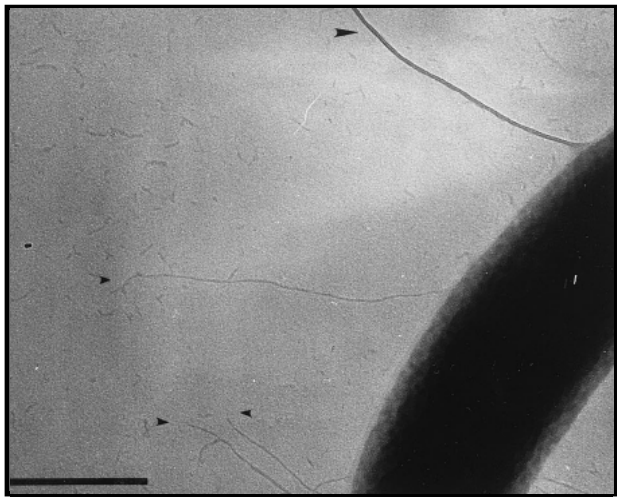


Figure 1.10- Electron microscopic image of pili on *L. pneumophila*, negatively stained with 1% phosphotungstic acid, pH 6.5. Both short and long pili can be seen, and are indicated by small arrowheads. A notably thicker flagellum can also be observed, and is indicated by the larger arrowhead (Taken from Stone and Abu Kwaik, 1999; Bar represents 0.5 μm).

In addition to pilin biogenesis, the group found that the *Legionella pilB* gene was strongly related to the *pilB* gene of *Ps. aeruginosa*, which, along with its homologous genes in other organisms, is a constituent of type II protein secretion systems. It has been previously established that these genes are required for the assembly of type IV pili (Lauer *et al*, 1993) and that loss of these genes results in organisms which do not express pili, and are therefore dramatically impaired in their ability to adhere to host cells (Strom and Lory, 1993; Heckles, 1989).

Due to the genomic organisation of the *pilB, pilC* and *pilD* genes, the group believed that the genes may have been co-transcribed. The *pilC* gene was located 8 bp past the end of the *pilB* ORF, whilst the *pilD* gene was located only 48 bp past the end of *pilC*.

Liles *et al* (1999) examined the function of the *pilD* gene by mutating the *pilD* gene in *L. pneumophila* through insertional inactivation of a kanamycin-resistance cassette. The mutant strains were found to grow comparably to the parent strain in bacteriological media, however it was noted that at least three proteins were not secreted in the mutant strain. This indicated that the *pilD* gene was involved in the secretion of proteins. The *pilD* mutant also experienced difficulty growing within the amoebae *Hartmanella vermiformis*. This effect

was reversed upon complementation of the mutant with a functional *pilD* gene. This observation led to the conclusion that intracellular growth of *L. pneumophila* is reliant upon cellular proteins secreted by a type II secretion mechanism.

Overall therefore, the *pilD* gene is seen as an integral member of the *pilBCD* gene cluster. It is a prepilin peptidase that cleaves and methylates both pilins and pseudopilins that eventually assemble into type IV pili. These type IV pili have been shown to promote bacterial attachment to host cells, and have been correlated to DNA transformation competence of *L. pneumophila* (Stone and Abu Kwaik, 1999).
L. pneumophila proteins believed to utilise this type II system consist of a zinc metalloprotease, acid phosphatases, lipases, phospholipases A and C, and a lysophospholipase A (Aragon *et al*, 2000; 2001; 2002).

1.5.5 *H*eat *S*hock *P*rotein 60 (Hsp60)

Heat **s**hock **p**roteins (Hsp) are involved in bacterial biogenesis, and are highly conserved proteins which are expressed in virtually all eukaryotic and prokaryotic cells. They are also known as chaperones, or stress proteins, and their intracellular expression in the cell can be observed to markedly increase when cells are subjected to stresses such as nutrient deprivation, oxygen radicals, viral infection and heat shock (Retzlaff *et al*, 1994). There have been some recent studies that indicate that although Hsp are mainly intracellular proteins, some Hsp can be surface expressed and can be secreted extracellularly (Ensgraber and Loos, 1992., Evans et al, 1992)

In *Legionella*, the Heat shock protein is a 58 to 60 kDa protein which is believed to be expressed by all *Legionella* strains. The protein has been found to contain a genus-specific epitope which is recognised by monoclonal antibodies, as well as epitopes which cross-react with many gram negative bacterial species (Plikaytis *et al*, 1987). In *Legionella*, the Hsp is usually associated with the cytoplasmic membrane (Gabay and Horwitz, 1985), but may translocate to the cell surface when the bacteria are growing intracellularly (Hoffman *et al*, 1990).

The nucleotide sequence of the *Legionella* Hsp60 was elucidated by the group of Sampson *et al* (1990) and the protein was found to be homologous to the Hsp of other organisms including the GroEL protein of *E. coli*, the 65-kDa antigen of *Mycobacterium tuberculosis*, and the HtbB protein of *Coxiella burnetii* (Hoffman *et al*, 1989., 1990).

The role of Hsp60 in *Legionella* pathogenesis has been studied, but there has been no direct link made with Hsp60 and *Legionella* virulence. However, it has been suggested that *Legionella* Hsp60 expression may be upregulated when the organism is residing within the rather unfavourable conditions of the phagosome (Dowling *et al*, 1992). More recently, Fernandez *et al* (1996) utilised radiolabelled *L. pneumophila* cells and human monocytes to determine which proteins were expressed in the early stages of infection. They examined polypeptide profiles of both adherent and intracellular bacteria, and found that synthesis of the *Legionella* Hsp60 had significantly increased, along with the *Legionella* OmpS protein. The group then tried to determine whether the protein was involved in virulence by creating a Hsp60 mutant. Although they were not able to reach a clear conclusion, they did find that although Hsp60 is located in the periplasm of both virulent and avirulent *L. pneumophila*, it was only in virulent strains of the organism that significant amounts of Hsp60 were released into the phagosome vacuole, during the infection cycle. The group speculated that the increase in Hsp60 levels during intracellular growth, combined with the finding that Hsp60 is found extracellularly associated with the phagosome membrane, may mean that Hsp60 is required for maintaining *Legionella* spp. growth in the replicative phagosome. They also acknowledged that the *Legionella* Hsp60 may be involved in the assembly or secretion of other proteins or factors involved in the virulence of the organism.

Garduno *et* al (1998) tried to determine whether the Hsp60 of *L. pneumophila* played a similar role in adhesion to cells as the recently described Hsp70 of *H. influenzae*. The group analysed the role of Hsp60 in the adherence and invasion of *Legionella* using the non-phagocytic HeLa cell line. Interestingly, they found that the *L. pneumophila* Hsp60 promoted binding to the HeLa cells and aided in the subsequent internalisation of virulent *L. pneumophila.* However, the group also acknowledged that this observation may not be exclusively due to the action of Hsp60, as non-virulent *Legionella*, which were devoid of Hsp60 on their surface were still able to attach well to the HeLa cell line.

The Hsp60 of *Legionella* has also been determined to play a role in immune recognition, particularly by immune cells such as T lymphocytes. In patients with confirmed cases of legionellosis, T cells were found to proliferate in response to challenge with purified Hsp60

(Weeratna *et al*, 1994). In addition to this, Retzlaff *et al* (1994) found that infection of murine macrophages, with either *L. pneumophila* bacteria, or purified Hsp60, elicits the production of the cytokine interleukin 1b (IL-1b).

1.5.6 *M*ajor *O*uter *M*embrane *P*rotein (mOMP)

The **M**ajor **o**uter **m**embrane **p**rotein (mOMP) of *Legionella* spp. is a 24-29 kDa surface-exposed outer membrane protein (porin), which is considered to be the organisms' most abundant protein (Gabay and Horwitz, 1985).

Gabay and Horwitz (1985) demonstrated that the mOMP is a peptidoglycan-associated porin, which remains linked to peptidoglycan even following treatment of the cells with 2% SDS and 2 mM $MgCl_2$ at 60°C. This is in contrast to most other gram negative bacterial porins, which can usually be solubilised from the membrane with the use of detergents (Hancock, 1987).

Porins form channels through the outer membrane of gram negative bacteria, which allow hydrophilic, low molecular weight molecules to pass through (Nikaido and Vaara, 1985).

The mOMP of *L. pneumophila* is described as being similar to the *E. coli* K-12 Omp porins, OmpF and OmpC. However, in contrast to the *E. coli* porins, which dissociate completely from peptidoglycan at high concentrations of salt (Hasegawa *et al*, 1976), the mOMP of *L. pneumophila* only dissociates to about 50% under similar conditions (Gabay and Horwitz, 1985).

Several years after the study of Gabay and Horwitz (1985), Hoffman *et al* (1992) analysed the structure of the mOMP of *L. pneumophila*, and found that the protein is covalently bound to peptidoglycan via a modified 28-kDa subunit, and is cross-linked through interchain disulfide bonds to other 28-kDa subunits. This again is unlike most other porins, which are usually anchored noncovalently into the peptidoglycan of the cells' outer membrane (Hancock, 1987).

The difference in molecular weight of the *L. pneumophila* mOMP observed by different groups is believed to be due to the different isolation methods employed for extraction of the protein. For example, groups using HEPES buffer (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) find that the mOMP has a molecular weight of 29 kDa (Hindahl and

Iglewski, 1984). In contrast to this, groups using Tris buffer find the mOMP to be a 24 kDa protein (Engleberg *et al*, 1984).

Bellinger-Kawahara and Horwitz (1990) previously analysed mechanisms of *L. pneumophila* phagocytosis via complement fixation. They found that monocyte complement receptors CR1 and CR3, and the serum complement component C3, mediate the phagocytosis of *L. pneumophila.* Further to this, they now examined C3 fixation to *L. pneumophila*, to identify acceptor molecules for C3 on the surface of *L. pneumophila*. This was achieved using whole-cell enzyme-linked immunosorbent assays (ELISA) to evaluate C3 fixation to the surface of bacteria. They found that C3 fixation takes place via the alternative pathway of complement activation. They also found that C3 fixes selectively to specific acceptor molecules on the cell surface. The group acknowledged that theoretically, this could be any surface bound protein or LPS molecule with exposed and accessible COOH or NH_2 groups. They found however, that in nonimmune serum, it is the mOMP of *L. pneumophila* which is the molecule specifically targeted for C3 fixation.

For *L. pneumophila*, two mechanisms have been described for attachment to host cells. One of these is opsonin-dependant binding (Horwitz, 1993), whilst the other is opsonin-independant (Gibson *et al*, 1994). For the opsonin-dependant system, it is complement receptors CR1 and CR3 on human phagocytes which recognise the complement fragments C3b and C3bi, which ultimately leads to the phagocytosis of organisms, including *Legionella*. As discussed above, the group of Bellinger-Kawahara and Horwitz (1990) determined that it is the mOMP of *L. pneumophila* which selectively binds the C3 complement component, which in turn leads to uptake of the organism by complement-mediated phagocytosis. Krinos *et al* (1999) therefore decided to further analyse the role of the mOMP in *Legionella* adhesion to host cells. The group achieved this by creating an *E. coli* clone which contains a plasmid encoding the mOMP gene of *Legionella*. The *E. coli* strain overexpressing the *L. pneumophila* mOMP was five times more adherent to U-937 cells than the parent *E. coli* strain, in the absence of opsonins. The group therefore concluded that the mOMP of *L. pneumophila* also appears to play an important role in opsonin-independent binding of the organism to U-937 cells. They acknowledged, however, that further work is required to fully determine the role of the mOMP in the pathogenesis of *L. pneumophila*.

1.5.7 Peptidoglycan-associated Lipoprotein (Ppl)

The peptidoglycan-associated lipoprotein (Ppl) of *L. pneumophila* is an outer membrane protein which has a predicted mass of 19 kDa. The protein has been found to be immunogenic, and is linked to the peptidoglycan layer of the bacterium (Engleberg *et al*, 1991).

Hindahl and Iglewski (1987) cloned and expressed the *L. pneumophila ppl* gene, and performed distribution studies to determine the prevalence of the gene within the genus *Legionella*. They included *L. pneumophila* serogroups 1-8, and the *Legionella* species of *L. bozemanii*, *L. dumoffii*, *L. gormanii*, *L. longbeachae* and *L. micdadei.* Western blotting results with anti-Ppl antibodies revealed reactivity with all of *Legionella* serogroups 1-8, and all *Legionella* species, except for *L. gormanii*.

Later, Ludwig *et al* (1991) also cloned and sequenced the DNA fragment encoding the Ppl protein of *L. pneumophila*, and found that the gene is located on a 1.8 kb *Cla*I fragment. The *ppl* gene was found to encode a protein product of 176 amino acids, with an estimated molecular mass of 18.9 kDa. The group found that the protein product of the *ppl* gene contains a lipoprotein signal sequence cleavage site between amino acids 21 and 22, and cleavage here results in a mature protein of 155 amino acids, and a predicted mass of 16.8 kDa (Wu and Tokunaga, 1986). The N-terminal region comprises of a hydrophilic region followed by an intensely hydrophobic region, and the sequence correlates with reported membrane protein signal sequences.

The *ppl* gene showed a high degree of similarity with the peptidoglycan-associated lipoprotein (PAL) genes of *H. influenzae* and *E. coli* K-12 (Ludwig *et al*, 1991).

The PAL of *E. coli* is described as being complexed with the Tol system. This Tol-PAL system is said to consist of several proteins that form two complexes. One of these is located in the cytoplasmic membrane, and consists of the TolA, TolQ, and TolR proteins. The other is associated with the outer membrane and consists of PAL and the periplasmic protein TolB (Bouveret *et al*, 1995). Overall, these *tol-pal* genes are believed to be involved in the maintenance of outer membrane integrity, and deletion of these genes results in the formation of membrane vesicles, which is indicative of a cell envelope assembly fault

(Bernadac *et al*, 1998). These results can be strengthened by the findings of Rodriguez-Herva *et al* (1996) who found that the PAL lipoprotein of *Pseudomonas putida* also served the function of maintaining the integrity of the cell envelope of the organism.

Recently, Hellman *et al* (2000; 2002) made the interesting finding that the PAL is one of three *E. coli* proteins, in addition to LPS, which is released by bacterial cells following incubation with human serum, and which may be involved in the development of gram-negative bacterial sepsis. They found that very small amounts of purified PAL was able to stimulate the macrophages of C3H/HeJ mice, and that injection of PAL into these mice stimulated the production of serum cytokines, and increased the expression of pulmonary and myocardial inflammatory markers. They also found that *E. coli* with reduced or truncated PAL lipoprotein expression was less virulent than the wild-type bacterium. From this, the group concluded that the PAL of *E. coli* may indeed be involved in mediating gram negative bacterial sepsis.

Kim *et al* (2003) recently analysed the potential of the *L. pneumophila* PpL for use in a urinary based detection system. They raised antibodies to *L. pneumophila* serogroup 1 purified PAL, and then analysed the potential of the resulting IgG antibodies to detect urinary PAL antigen from infected guinea pigs, using ELISA assays. The group tested 17 urine samples from infected guinea pigs, along with 67 negative control samples. From this, they found that the assay had a specificity rate of 88.2% and a sensitivity rate of 95.5%. As a control, the group also included 161 human urine specimens from adults with either non-*Legionella* pneumonia, or with a urinary tract infection, and found that none of these gave a positive result with the ELISA.

Overall, due to the ease and stability of producing the recombinant Ppl lipoprotein, and the promising specificity and sensitivity results, the group concluded that the Ppl protein demonstrated considerable potential for the development of a diagnostic immunoassay for *L. pneumophila* detection (Kim *et al,* 2003).

1.6 Summary

Legionella continues to be a problematic organism world-wide, and although our knowledge of its pathogenic mechanisms is quite comprehensive, there is still a great deal which remains to be elucidated. *Legionella* outer membrane proteins, in particular, often contribute greatly to the virulence of the organism. As they are located on the outer surface of the bacterium, they are often a first point of contact for bacterial cell attachment to host cells and tissues. They also serve as a channel through which bacterial components can be excreted outside the cell, such as toxins or effector molecules, or imported into the cell from the surrounding environment (Nikaido, 2003).

There may be numerous other outer membrane proteins which are yet to be discovered, that play an important role in *Legionella* pathogenesis. Understanding the function and role of these proteins may help to further clarify the process of disease development and enable the development of better *Legionella* prevention and control measures.

1.7 Aims of thesis

The outer membrane of *Legionella* will be analysed for novel proteins. This will be performed both through immunological techniques, and bioinformatics tools such as the sequence analysis program, Prosite. Novel proteins identified will then be characterised in more detail.

CHAPTER II

Materials and Methods

2. General procedures

Glasswares, liquid and agar media, pipette tips, PCR tubes and general materials were sterilised by autoclaving at 121^{o}C (220 kPa) for 20 min. All chemicals used were of analytical laboratory reagent grade. All solutions were prepared using deionised water obtained from a Millipore Milli-Q water system (Liquipure, Melbourne, Australia).

Centrifugation of volumes smaller than 1.5 ml were performed in an EBA12 Microcentrifuge (Hettich-zentrifugen). Centrifugation of volumes between 1.5 ml to 50 ml were performed using a Beckman Allegra^{TM} 21R Centrifuge. Centifugation of volumes larger than 1.5 ml, requiring speeds above 6,000 x *g* were performed in a Beckman JA21 M/E Centrifuge.

Media containing antibiotics or supplements were autoclaved and then allowed to cool to 50^{o}C before the addition of antibiotics or supplements and were dispensed into petri dishes under a flame or in a laminar flow cabinet. The media plates were then dried at 37^{o}C for 1 hr before use.

All glasswares were washed in Pyroneg detergent (Diversey-Lever Pty. Ltd., Australia), rinsed twice in tap water and then twice in deionised water.

Solutions were dispensed using either; a Finnpipette® 0.5-10 µl (Labsystem) pipette for volumes ranging from 0.5 µl to 10 µl; or a Finnpipette 5-40 µl (Labsystem) pipette for volumes ranging from 10 µl to 40 µl; or a Gilson P-200 Pipetman Micropipette (John Morris Scientific, Australia) for volumes ranging from 200 µl to 1000 µl; or a Finnpipette 1000-5000 µl (Labsystem) for volumes ranging from 1000 µl to 5000 µl.

2.1 Materials

2.1.1 General chemicals and equipment

ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit	Perkin-Elmer Corp., U.S.A
Acetic acid, glacial	BDH Chemicals, U.K.
Agar (Bacteriological Agar No. 1)	Oxoid Ltd., U.K.
Agarose (DNA grade)	Progen Industries, Australia
Albumin, bovine serum (BSA)	Sigma Chemical Co., U.S.A
Ammonium hydroxide	BDH Chemicals, Australia
Ampicillin	CSL Ltd., Australia
Balances:	
- Analytical balance	Sartorius Gottingen, Germany
- Balance (0.1-500g)	U-Lab, Australia
Benzamidine	Sigma Chemical Co., U.S.A
5-bromo-4-chloro-3-indoyl-b-D-galactopyranoside (X-gal)	Diagnostic Chemicals Ltd., Australia
Bromophenol blue	BDH Chemicals, Australia
Centrifuges	
- Eppendorf centrifuge	Eppendorf Geratebau, Germany
- Bench top centrifuge	Centaur 2, MSE
- High speed centrifuge	Beckman, U.S.A
- Ultra centrifuge	Beckman, U.S.A
Centrifuge tubes	
- 1.5 ml centrifuge Eppendorf tubes	Treff AG, Switzerland
- 10 ml centrifuge tubes	Sarstedt, Australia
- 50 ml centrifuge tubes	Greiner Labortechnik, Germany
Chloroform	Ajax Chemicals, Australia
4-Chloro-1-napthol	Sigma-Aldrich Pty. Ltd., U.S.A
Cover slips	Mediglass, Australia
Cryovials (1.8 ml)	Nalgene, U.S.A

DIG DNA Labelling and Detection Kit	Boehringer Mannheim, Germany
DNA Ligase (T4) and Ligase buffer	Boehringer Mannheim, Germany
DNA Polymerase (AmpliTaq)	Perkin Elmer, U.S.A
Deoxynucleoside triphosphates (dNTP's) (10mM)	Boehringer Mannheim, Germany
Electrophoresis Power Supply:	
- EPS 500/400	Pharmacia LKIB, Sweden
- EPS 3000xi	Biorad Laboratories, U.S.A
- EPS 600	Pharmacia LKIB, Sweden
Electrophoresis Units:	
- DNA	
(i) Mini gel (GNA-100)	Pharmacia LKIB, Sweden
(ii) Midi-gel (wide mini-sub cell GT)	Biorad Laboratories, U.S.A
(iii) Maxi-gel (GNA-200)	Pharmacia LKIB, Sweden
- Protein	
(i) Mini Protean III gel system	Biorad Laboratories, U.S.A
(ii) Maxi Protean gel system	Biorad Laboratories, U.S.A
Electroporation apparatus	Biorad, U.S.A
Ethanol	BDH Chemicals, U.K
Ethidium Bromide (EtBr)	Boehringer Mannheim, Germany
Ethylenediamine tetra acetic acid, disodium salt (EDTA)	BDHChemicals, Australia
Filter (acrodisc 0.2 mm, 0.45 mm)	Gelman Sciences, U.S.A
Fluorescent Microscopy Unit	
- Camera	Panasonic CCTV (WV-BP332E)
- Microscope	Olympus EX5IWI
- Burner unit	Olympus U-RFL-T
- Control unit	Olympus TH4-200
Gel Documentation UV transilluminator	Gel Documentation^{TM,} Biorad, Australia
Glycerol	BDH Chemicals, U.K
HEPES buffer	Cytosystems Pty. Ltd., Australia
Hexadecyltrimethyl ammonium bromide	Sigma-Aldrich Pty. Ltd., U.S.A

(CTAB)	
Hydrochloric acid (32%)	Ajax Chemicals Ltd., Australia
Imidazole	Sigma Chemical Co., U.S.A
Isoamyl alcohol	BDH Chemicals, Australia
Isopropanol	Ajax Chemicals Ltd., Australia
Isopropyl-thiogalactoside (IPTG)	Sigma Chemical Co.., U.S.A
Lambda DNA	Pharmacia LKIB, Sweden
Leupeptin	Sigma Chemical Co., U.S.A
Lysozyme	Boehringer Mannheim, Germany
Magnesium chloride	BDH Chemicals, U.K
2- Mercaptoethanol (β-mercaptoethanol)	Biorad, U.S.A
Methanol	BDH Chemicals, U.K
Microscopes	
- Light microscope	Olympus Optical Co., Japan
- Transmission electron microscope EM100SX-1	Jeol Ltd., Japan
Microscope slides	LOMB Scientific Co., Australia
Molecular weight markers (protein)	
- Prestained	SeeBlue®, Invitrogen, U.S.A
- Unstained	Fermentas Life Sciences, U.S.A
Needle (19g, 21g, 26g)	Terumo Pty. Ltd., Australia
Nitrocellulose membrane (Hybond-N)	Amersham U.S.A
Orbital shaker	Chiltern orbital shaker SS60
Paraffin	BDH Chemicals, Australia
Petri Dish	Nunc, Denmark
pH Meter	Radiometer, Denmark
Phenol/Chloroform	BDH Chemicals, Australia
Phosphate Buffered Saline (PBS)	Oxoid Ltd., U.K
Potassium Acetate	BDH Chemicals, Australia
Potassium dihydrogen orthophosphate	BDH Chemicals, Australia
Proteinase K	Sigma Chemical Co.., U.S.A
QIAEX II Gel Extraction Kit	Qiagen, Clifton Hill, Victoria

Restriction enzymes	New England, Australia Pharmacia, Australia Promega, Australia
RNase	Boehringer Mannheim, Germany
Skim milk	Bonlac Foods Ltd., Australia
Sodium Acetate	BDH Chemicals, U.K
Sodium chloride	BDH Chemicals, U.K
Sodium Dodecyl Sulphate (SDS)	BDH Chemicals, U.K
di-Sodium hydrogen orthophosphate (anhydrous)	BDH Chemicals, Australia
Sodium chloride (pellets)	BDH Chemicals, U.K
Sodium phosphate	Mallinckrodt Inc., U.S.A
Sonicator	Branson Sonic Power Co., U.S.A
Sucrose	BDH Chemicals, U.K
Syringe (1ml, 5ml, 10ml, 20ml, 50ml)	Terumo Pty, Ltd., Australia
3,3',5,5'- tetramethylbenzidine (TMB)	BD Biosciences, U.S.A
N',N',N',N'- tetramethylethylenediamine (TEMED)	Biorad Laboratories, U.S.A
Trans-blot electrophoretic transfer cell	Biorad Laboratories, U.S.A
Transilluminator (UV)	Novex Australia Pty. Ltd.
(hydroxymethyl) aminomethane (Tris)	Boehringer Mannheim, Germany
Triton –X-114	Sigma-Aldrich Pty. Ltd., U.S.A
Tryptone	Oxoid Ltd., U.K
Tween 20	Sigma Chemical Co., U.S.A
Waterbath	Ratek Instruments (U-Lab, Melbourne, Australia)
Whatman blotting paper	Whatman, U.K
Wizard PCR DNA Purification Kit	Promega, U.S.A
Yeast Extract	Oxoid Ltd., U.K

2.1.2 Bacteriological Media

All media were prepared according to the manufacturers directions unless noted in the text.

Buffered Charcoal Yeast Extract (BCYE-α) Agar: 10 ml of warm (50°C) sterile distilled water was aseptically added to one vial of *Legionella* BCYE Growth Supplement (Oxoid Ltd, U.K). The vial contents was gently mixed and added to 90 ml of sterile *Legionella* CYE Agar base (Oxoid Ltd, U.K), cooled to 50°C. The mixture was then poured into sterile Petri dishes.

Buffered Yeast Extract Broth (BYE) broth: This method was based on that of Ristroph *et al* (1980). The contents of one vial of *Legionella* BCYE Growth Supplement (Oxoid Ltd, U.K) was reconstituted with 50 ml of warm (50°C) sterile distilled water. The vial contents was gently mixed and added to 450 ml of warm (50°C) sterile distilled water. 5 g of yeast extract was then added to the mixture. The broth was then filter sterilised through a 0.45 µm filter (Gelman Sciences, U.S.A), and the pH was adjusted to 6.9.

Glycerol freezing medium: 50 % (v/v) glycerol (DBH), 50 % (v/v) HIB (Difco).

Luria agar (LBA): 1% (w/v) yeast extract (Oxoid Ltd, U.K), 0.5% (w/v), tryptone (Oxoid Ltd., U.K), 0.5% (w/v) sodium chloride (NaCl; May & Baker Australia (M&B) and 1% (w/v) bacteriological agar (Oxoid Ltd., U.K).

Luria broth (LBB): 1% (w/v) yeast extract (Oxoid Ltd, U.K), 0.5 % (w/v), tryptone (Oxoid Ltd., U.K), 0.5% (w/v) sodium chloride (NaCl; May & Baker Australia (M&B)).

SOC broth (SOC): 2% (w/v) tryptone (Oxoid), 1% (w/v) yeast extract (Oxoid), 10 mM NaCl (M&B), 2.5 mM potassium chloride (KCl) (British Drug House (BDH), Australia), 10 mM $MgCl_2$ (BDH), 10 mM magnesium sulphate ($MgSO_4$) (BDH) and 20 mM glucose (BDH).

X-Gal/IPTG agar: Solidified sterile LA supplemented by spreading on agar plates. 5-bromo-4-chloro-3-indoyl-β-D-galactose (X-gal) (Diagnostic Chemicals Limited (DCL),

Australia) and isopropyl-β-D-thiogalactopyranoside (ITPG) (DCL) added to a final concentration of 32 μg/ml. LA plates containing appropriate antibiotics were also supplemented with X-gal and ITPG in the same manner.

2.1.3 Solutions

Acetic acid, glacial: 5%, 80%, 100% glacial (BDH).

Acrylamide/bisacrylamide solution: A 29.2% (w/v) acrylamide and 0.8% (w/v) bisacrylamide.

Agarose: 1% DNA grade agarose (Progen) in 1×TAE buffer.

Ammonium acetate: 10 M ammonium acetate (NH_4Ac) (BDH).

Ammonium persulphate (APS): 10% (w/v) ammonium persulphate (Bio-Rad) in distilled water and freshly prepared before use.

Bovine serum albumin fraction V (BSA): 1 mg/ml in Milli-Q water, stored at –20°C (Sigma).

Bromophenol blue: 1% (w/v) in Milli-Q water, stored at RT (Sigma).

Butanol: 99.5% Butan-1-ol (BDH).

Cell lysate buffer (for the preparation of whole cell lysates): 0.1 M Tris-base (BM), 2% (w/v) SDS (BDH), 15% (v/v) glycerol (BDH) and 2 mM PMSF (Sigma), pH 6.8.

Chloroform: 100% (v/v) chloroform (BDH).

4-chloro-1-napthol: 3 mg/ml 4-chloro-1-napthol (Sigma) in methanol, freshly prepared before use.

CI: 96% (v/v) chloroform, 4% (v/v) isoamyl alcohol (BDH).

Coomassie blue stain: 0.05% (w/v) Coomassie brilliant blue R-250 (Biorad), 50% (v/v) methanol (BDH), 10% (v/v) glacial acetic acid.

CTAB/NaCl: 4.1 g of NaCl were dissolved in 80 ml of H_20 and 10 g of CTAB were added while heating at 65°C (Sigma).

Destain solution (for Coomassie blue stain): 40 % (v/v) ethanol (BDH) and 5 % glacial acetic acid (BDH).

DNA denaturation solution: 0.5 M NaOH, 1.5 M NaCl in Milli-Q® water.

EDTA solution: pH 8.5, 1 mM, 0.25 M and 0.5 M EDTA (BDH) in Milli-Q® water.

Ethanol: 70%, 95%, 100 % ethanol (BDH) in Milli-Q® water.

Ethidium bromide (EtBr): 10 mg/ml EtBr (BDH) in Milli-Q® water.

Gel-buffer: 3.0 M Tris, 0.3% SDS, pH 8.45 in Milli-Q® water.

Glycerol: 50 % (v/v) glycerol (BDH) in Milli-Q® water.

Iso-Propanol: 99.5 % Propan-2-ol (BDH).

IPTG: 2 g IPTG (Sigma) in 10 ml H_2O, filter-sterilised, dispensed into 1 ml aliquots and stored at –20°C, used at a final concentration of 100 mM.

KOAc: 60 ml 5M KOAc, 11.5 ml HOAc, 28.5 ml H_2O.

Laemmli sample mix: 1.25 ml 2M Tris-HCl, pH 6.8, 8 ml 10% SDS, 4 ml 100% glycerol, 2 ml β-mercaptoethanol, 0.16 ml 0.5% bromophenol blue, made up to 30 ml with water

Lowry reagent A: 4% $CuSO_4 \cdot 5H_2O$ in Milli-Q® water, stored at RT.

Lowry reagent B: 2% Na_2CO_3, 4% NaOH, 0.16% sodium tartrate, 1% SDS, stored at RT.

Lowry reagent D: folin-ciocalteau (BDH) reagent was mixed 1:1 with distilled water, freshly prepared before use.

Methanol: 100% (BDH).

PCI: 50% saturated phenol, 48% chloroform, 2% isoamylalcohol (BDH)

Phenol: Phenol saturated with TE buffer, pH 8.0 (BDH).

Phosphate-Buffered Saline (PBS): PBS solution was prepared by dissolving 1 PBS tablet (Oxoid) in 100 ml Milli-Q® water.

SDS: 10% (w/v) SDS (BDH).

SDS-PAGE Reservoir buffer stock (10x): 0.25M Tris-HCl, 1.92M glycine (Sigma), 1% (w/v) SDS, adjusted to pH 8.3. This solution was stored at 4°C.

SDS-PAGE Resolving gel (12.5%): 3.125 ml 40% (w/v) acrylamide-bisacrylamide 29:1 solution (Amresco), 1.25 ml of 8x resolving gel buffer stock, 100 µl 10% (w/v) SDS, 5.025 ml MilliQ® H_2O. Just before pouring, 500 µl 1.5% (w/v) ammonium persulphate (Biorad) and 15 µl N,N,N',N'-tetramethylethylenediamine (TEMED) (Biorad) was added to the mixture.

Resolving gel buffer stock (8x): 3.0M Tris-HCl pH 8.8. This solution was stored at 4°C.

SDS-PAGE Stacking gel (3.75%): 375 µL 40% (w/v) acrylamide-bisacrylamide 29:1 solution, 1ml of 4x stacking gel buffer stock, 40µL 10% (w/v) SDS, 2.385 ml MilliQ® H_2O. Just before pouring add 200 µL 1.5% (w/v) ammonium persulphate and 10 µL TEMED.

Stacking gel buffer stock (4x): 0.5M Tris-HCl pH 6.8. This solution was stored at 4^{o}C.

Sodium hydroxide: 10 M, 0.1 M NaOH (BDH).

Solution I (for plasmid isolation): 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0, 4 mg/ml lysozyme.

Solution II (for plasmid isolation): 1% (w/v) SDS and 0.2 M NaOH, freshly prepared.

Solution III (for plasmid isolation): 3 M KOAc (295 g/L) (BDH), 2 M HOAc (115 ml glacial acetic acid/L) (BDH).

Stop solution (loading dye) 10×: 10% Ficoll (BDH), 50% glycerol, 0.5% orange G, 1% SDS, 10 mM EDTA, 50 mM Tris/HCl (pH 8.0).

TAE buffer: TAE buffer was made up as a 50× stock and diluted to 1× before use. For preparation of 50× TAE stock: 24.2% (w/v) Tris-base (BM), 55.17% (v/v) glacial acetic acid (BDH) and 1.86% (w/v) EDTA (BDH) were dissolved in Milli-Q® water.

TE buffer: 10 mM Tris-base (BM), 1 mM EDTA (BDH), pH 8.0.

TE/RNase buffer: 20 μg/ml pancreatic ribonuclease (RNase) in TE buffer, was boiled for 10 min and left to cool slowly.

TEG: 25 mM Tris/HCl pH 8.0, 10 mM EDTA, 50 mM glucose was sterilized by autoclaving.

Western Transfer buffer: 12 mM Tris, 192 mM glycine, 10% (v/v) methanol.

2.1.4 Antibiotic stocks

Ampicillin: 500 mg/vial (Centrafarm) was dissolved in 5 ml sterile water to give a 100 mg/ml stock solution. Stored at –20^{o}C.

Kanamycin: Kanamycin (Sigma) was made up as a stock solution of 100 mg/ml in sterile water, and was stored at –20^{o}C.

2.1.5 Enzyme stocks

Lysozyme: 4 mg/ml was made freshly before use (Roche).

Pfu DNA polymerase: 3 U/µl (Promega).

Proteinase K: 10 mg/ml stock solution, stored at –20°C (BM).

Restriction enzymes: all restriction enzymes used were purchased from Promega (Sydney, Australia), New England (Queensland, Australia), Pharmacia (Sydney, Australia), and were used as recommended by the manufacturer.

DNase (RNase-free): RQ1 RNase-free DNase with 10× buffer (Promega).

T4 ligase: 1 U/µL, stored at –20°C (BM).

Taq DNA polymerase: 5 U/µL AmpliTaq (Perkin Elmer).

2.2 General Methods

2.2.1 Bacterial methods

2.2.1.1 Bacterial strains and plasmids

All bacterial strains and plasmids used in this study are listed in individual chapters of this thesis.

2.2.1.2 Bacterial culture conditions

Legionella strains were routinely grown under aerobic conditions on BCYEα agar (Oxoid) for 48 hr at 37ºC. *E. coli* was grown in LB broth or agar at 37ºC overnight.

2.2.1.3 Storage of bacterial strains

Legionella strains were stored at –70ºC in cryovials (Nalgene, U.S.A) containing glycerol freezing medium for long-term storage. Other bacterial strains were stored at 4^{o}C on appropriate media, supplemented with the appropriate antibiotics where required, for short-term storage.

2.2.2 Methods for protein analysis

2.2.2.1 Preparation of whole cell lysates

Bacterial cells were harvested in 3.0 ml of 10 mM Tris-HCl (pH 7.0). The cell suspension was centrifuged at 5,000 × *g* for 5 min at RT and the bacterial pellet washed by resuspending in 3 ml of 10 mM Tris-HCl (pH 7.0) followed by centrifugation as above. The bacterial pellet was resuspended in 0.5 ml of cell lysate buffer and boiled for 5 min. Cell debris was removed by centrifugation at 10,000 × *g* for 2 min, and the supernatant was transferred to a new microfuge tube for protein determination and further use. All whole cell lysate samples were stored at –20ºC.

2.2.2.2 Determination of protein content (Lowry assay)

The protein content of samples was determined by a modification of the Lowry method as described by Markwell *et al* (1978). Eppendorf tubes were set up for both control samples and test samples. The set-up followed for control samples is shown in **Table 2.1**.

Table 2.1. Test tube set-up for the Lowry assay

Tube No	1	2	3	4	5	6	7	8	9	10
Standard Albumin (1 mg/ml) (µl)	0	10	20	30	40	50	70	100	0	0
Cell lysate (µl)	0	0	0	0	0	0	0	0	10	10
Milli-Q water (µl)	200	190	180	170	160	150	130	100	190	190

Six hundred µl of alkaline copper reagent (50 ml of 2% Na_2CO_3 , 0.4% NaOH, 0.16% sodium potassium tartrate and 1% SDS, mixed with 5 ml of 4% $CuSO_4.5H_2O$) was added each tube and these were allowed to stand at room temperature for 20 minutes. Sixty µl of Folin reagent (Folin-Ciocalteau reagent mixed at a 1:1 ratio with distilled H_2O) was added to each tube, the tube was then rapidly mixed and allowed to stand for 30 minutes. Two hundred µl from each tube was then aliquoted into a 96 well microtitre tray and the tray was read on an ELISA reader at 600nm. The ELISA reader then determined the concentration of unknown samples through the development of a standard curve, consisting of the absorbance versus protein content (µg).

2.2.2.3 Preparation of glycine SDS-PAGE gels

Glycine SDS-PAGE was performed using a continuous buffer system (Laemmli, 1970). Electrophoresis was conducted at 180 V in a Mini or Maxi Protein system in an electrophoresis tank (Bio-Rad) for 8-10 hr under denaturing or reducing conditions. Two gels were run simultaneously in this system. The composition of the maxi and mini stacking gel and resolving gels are given in **Table 2.2** and **Table 2.3.** One of the gels was used for transfer onto nitrocellulose membrane for immunoblotting analyses and the other gel was stained by Coomassie blue staining. Staining was performed for 1 hr, followed by destaining with several changes of destain solution.

Table 2.2 The composition of two maxi SDS-PAGE gels.

Gel components	4% stacking gels (ml)	12.5% resolving gels (ml)
40 %(w/v) acrylamide	1.6	15.6
3 M Tris-HCl + 0.3 % SDS, pH 8.45	3.0	16.5
100 % glycerol	-	5.2
Deionised water	7.4	12.7
10 % APS	0.1	0.2
TEMED	0.01	0.02
Total volume	12 ml	50 ml

Table 2.3 The composition of two mini SDS-PAGE gels.

Gel components	4 % stacking gels (ml)	12.5 % resolving gels (ml)
40 %(w/v) acrylamide	0.6	3.125
3 M Tris-HCl + 0.3 % SDS, pH 8.45	1.5	3.3
100 % glycerol	-	1.04
Deionised water	3.7	2.5
10 % APS	0.05	0.05
TEMED	0.005	0.005
Total volume	6.3 ml	10 ml

2.2.2.4 Electrophoretic transfer

The protein bands from SDS-PAGE gels were transferred onto a nitrocellulose membrane as described by Towbin *et al* (1992). The separating gel was removed and soaked in transfer

buffer to equilibrate for 5-10 min. A piece of nitrocellulose membrane, eight pieces of 3M Whatman paper and scotch brite pads were also soaked in transfer buffer for 5 min. The transfer cassette was made in the following order: 1 soaked scotch brite pad, 4 sheets of Whatman 3M paper, polyacrylamide gel, membrane, 4 soaked sheets of filter paper and then scotch brite pad on the back section of the cassette. The cassette was closed and submerged in a transblot tank with the membrane on the anode side of the gel. The electrophoretic transfer was done at constant voltage of 70 V for 2 hr.

2.2.2.5 Immunoblotting

Immunoblotting was performed by the method described by Maniatis *et al* (1982). After electrophoretic transfer, the region of nitrocellulose membrane surrounding the bound protein was blocked by incubation with 5 % skim milk in tris-buffered saline/Tween20 (TST) for 1-2 hr on a rotary shaker at RT. The skim milk solution was removed and the membrane washed once by gentle shaking in TST for 5 min. The membrane was then incubated with diluted primary antiserum (see individual chapters for details) overnight at 4 °C or 1 hr at RT on a rotary shaker. The membrane was then washed 3 times in TST buffer for 2-3 min. Secondary antibody, conjugated with horseradish peroxidase (HRP) immunoglobulin (Biorad) was then used, diluted in TST containing 1 % skim milk (see individual chapters for details). The bound peroxidase was visualised with 4-chloro-1-napthol. The SeeBlue ^{TM} Plus2 Pre-Stain standard (Invitrogen) was used as a molecular weight marker (**Appendix 1**).

2.2.3 DNA molecular techniques

2.2.3.1 Agarose gel electrophoresis for DNA size determination

Before electrophoresis, a 1/10 volume of 10 x loading dye was added to each DNA sample. DNA sizes were determined by running each sample against λ DNA standards prepared by *PstI* restriction enzyme digestion, on a horizontal 1-2 % (w/v) agarose (Progen, Aust.) slab gel (made up with 1x TAE buffer) in either midi (Pharmacia, Aust.) or mini (Bio-Rad) gel tanks. The DNA fragments were electrophoretically separated at 80 V for 2 hr. DNA size fragments generated by *Pst*I restriction enzyme digestion were: 11.5, 5.1+4.7+4.5, 2.8,

2.6+2.5+2.4, 2.14, 1.99, 1.70, 1.16, 1.09, 0.80, 0.51, 0.47, 0.45, 0.34, 0.264, 0.247, 0.216+0.211 kbp (**Appendix 2**).

Following electrophoresis the gel was stained in an EtBr-bath (0.5 μg/ml) for 5 minutes and destained in tap water for at least 30 minutes. The DNA fragments were then visualized using a Gel doc UV-transilluminator (Gel doc^{TM}, Biorad, U.S.A).

2.2.3.2 Quantification of DNA concentration

For plasmid DNA, DNA concentrations were estimated by comparison of sample DNA against the λ *Pst*I digested MW marker (50 μg/ml).

For quantitating the amount of the DNA, a reading from the spectrophotometer (Shimadzu UV160 Visible Recording spectrophotometer) was taken at wavelengths 260 nm and 280 nm. 1 ml of diluted DNA sample (1/100 dilution) was used to determine the concentration of DNA. An OD of 1 corresponded to 40 μg/ml for chromosomal DNA. Furthermore, the purity was determined by the ratio OD_{260} /OD_{280} > 1.8 for clean DNA, and 1.5 for 50 % protein.

2.2.3.3 Plasmid DNA extraction (mini-prep)

Plasmid DNA was isolated from the host bacteria using the mini-prep procedure of Ausubel *et al* (1995). Two ml of media was inoculated and grown at 37^{o}C overnight. 1.5 ml bacterial culture was transferred to a 1.5 ml eppendorf tube (Sarstedt 1.5 ml micro-tube), centrifuged for 2 min at 5,400 x *g*, the pellet was then resuspended in 100 μl TEG/lysozyme and vortexed. The tubes were left at RT for 5 min and 200 μl freshly prepared 0.2 M NaOH/0.1 % SDS solution was added to the tube and mixed. The tubes were left on ice for 5 min and 150 μl of 3 M cold potassium acetate (KOAc) was added and left on ice for another 5 min. Tubes were centrifuged and the supernatant transferred to clean eppendorf tubes. Fifty microliters of P/C/I was added to each tube, mixed and pelleted. This step was repeated with C/I instead of P/C/I. The supernatant was then transferred to a clean eppendorf tube and 900 μl 98% analytical ethanol was added to the tube and held at RT for 5 min. The tube was centrifuged for 5 min at 5,400 x *g* and the DNA pellet was washed with 1 ml 70%

ethanol. The tube was centrifuged again and the pellet air-dried. Plasmid DNA was finally resuspended in 30 µl Milli-Q® water with RNase and kept at -20°C.

2.2.3.4 Chromosomal DNA preparation

DNA was isolated from pure cultures by the CTAB procedure (Ausubel *et al.*, 1995). Briefly, one lawn plate of bacterial cells was grown overnight, harvested in 9.4 ml TE buffer and 0.1 ml 0.5 M EDTA and lysed with 0.5 ml 10 % (w/v) SDS. Proteinase K was added to a final concentration of 0.1 mg/ml and the mixture incubated at 37ºC for 2 hr. Then 1.8 ml 5 M NaCl and 1.5 ml 10% (w/v) CTAB in 0.7 M NaCl was added and the mixture incubated for 30 min at 65ºC. Five microliters of 24:1 chloroform/isoamyl alcohol was added and the mixture centrifuged for 10 min at 4ºC. The aqueous layer was removed and 5 ml of 25:24:1 phenol/chloroform/isoamyl alcohol was added. The aqueous layer was removed after centrifugation. The DNA was precipitated with 0.6 volume isopropanol and dissolved in water.

2.2.3.5 DNA ligation

Ligations were performed in the ratio of 1 vector molecule to 2 insert molecules, and 1 vector molecule to 4 insert molecules, with 10 U of T4 DNA Ligase and T4 DNA Ligase buffer. The reaction mix was made up to 20 µl with sterile Milli-Q® water and incubated at 16ºC overnight. The mixture was then used for transformation of *E. coli* DH5α cells, unless otherwise stated.

2.2.3.6 Phosphatase treatment of vectors

Plasmids were alkaline phosphatase treated by incubating 1 µg of restriction digested plasmid DNA with 0.05 U of calf-intestinal phosphatase in the appropriate buffer for 1 hour. The reaction was stopped by adding 0.5M EDTA and heat inactivating the enzyme at 65°C for 15 minutes.

2.2.3.7 Preparation of Competent cells

E. coli strains were grown overnight in 2 ml LBB in a shaking 37°C incubator. The fresh overnight culture was used to inoculate 200 ml LBB in a 500 ml flask which was then incubated for 3 h at 37°C with vigorous shaking. The culture was chilled for 30 min on ice before centrifugation in 50 ml tubes for 15 min at 3,400 x *g* at 4°C. The supernatant was discarded and the pellet resuspended in 50 ml sterile de-ionised H_2O and re-centrifuged. After discarding the supernatant, the pellet was resuspended in 25 ml sterile de-ionised H_2O and centrifuged again. The pellet was resuspended in 1 ml 10% (v/v) glycerol and centrifuged again. After resuspending in 0.75 ml 10% (v/v) glycerol, the pellet was stored at -70°C in aliquots of 85 µl.

2.2.3.8 Electrotransformation

Forty µl of competent cell suspension was mixed with DNA in a cold 1.5 ml polypropylene tube and stored on ice for 1 min before transferring to a cold 0.1 cm electroporation cuvette. The Gene Pulser apparatus (Bio-Rad) was set at 25 µF and 1.25 kV with the Pulser Controller set at 200 Ω. The mixture was pulsed once at these settings and immediately after pulsing 1 ml of SOC was added. The mixture was then transferred to a 1.5 ml polypropylene tube and incubated at 37ºC for 1 hr. A 100 µl volume of this suspension was plated out on agar plates containing selective antibiotics.

2.2.3.9 Polymerase Chain Reaction (PCR)

PCR was performed either by using isolated DNA or bacterial cells. PCR on fresh bacterial cells was performed by the following: fresh colonies grown on agar plates were picked up with sterile toothpicks. The toothpick was then put into a 50 µl PCR reaction mix (1 x reaction buffer, 15 µM $MgSO_4$, 2.5 U Taq DNA polymerase, 200 ng forward and reverse primer) and ground on the wall of the PCR tube for 2 seconds. Then the bacterial cells on the toothpick were subcultured on an agar plate with appropriate antibiotic. The PCR reaction included an initial denaturation of DNA at 94ºC for 1 min and then 35 cycles of consecutive

denaturation (94ºC for 30 s), primer annealing (57ºC for 30 s), and chain extension (72ºC, and the duration is based on a rate of 1 kbp/min,). The final elongation step was 10 min at 72ºC. Other PCR polymerases and reaction profiles are given in individual chapters.

2.2.3.10 Restriction enzyme digestions

Restriction enzyme digestions were performed according to the manufacturer's instructions. However, for every μg of DNA, 10 U or 20 U of restriction enzyme was used. Samples were incubated at 37ºC for 2 hours.

2.2.3.11 Southern blot analysis

2.2.3.11.1 Transfer of DNA to Nylon Membrane

Five μg of genomic DNA was digested with the desired restriction enzyme and was subjected to agarose gel electrophoresis. The DNA gels were then soaked in denaturation solution (0.5 M NaOH, 1.5 M NaCl) for 30 minutes followed by neutralisation solution (1.5 M NaCl, 0.5 M Tris-HCl, pH 7.0) for 30 minutes. The DNA was transferred to the nylon membrane by placing the membrane on top of the gel, followed by at least 4 sheets of 3M Whatman paper, approximately 30 sheets of paper towels, a glass plate and a 1 kg weight on top of the glass. The transfer was allowed to proceed overnight. The membrane was then washed once in 2 x SSC to remove any residual agarose from the membrane. The membrane was then air dried and wrapped in plastic sandwich wrap and placed on top of a UV transilluminator for 5 minutes to allow cross-linking of DNA to the membrane.

2.2.3.11.2 Labelling of probes with Digoxigenin

Labelling was performed using the DIG DNA Labelling and Detection kit (Roche Molecular Biochemicals). All procedures were carried out according to the manufacturer's instructions

using digoxigenin (DIG) –dUTP as the label. The labelled DNA probe was denatured before it was added to the membranes by boiling for 5 minutes.

2.2.3.11.3 DNA Hybridisation

The membrane was incubated at 60°C for 3 hours in hybridisation solution. The denatured probe was added to the hybridisation solution and the membrane was incubated overnight at 60°C.

2.2.3.11.4 Development of membranes

Membranes were washed twice in 2 x SSC containing 1 % SDS for 5 minutes at room temperature. This was followed by a wash in 2 x SSC plus 0.1 % SDS for 30 minutes at 65°C. Then a final wash with 0.1 % SSC containing 0.1 % SDS at 65°C for 15 minutes was performed. Positive hybridisations with DIG-labelled probes were immunodetected by incubating membranes with anti-DIG AP-conjugated antibodies followed by the addition of the substrate NBT/BCIP. All procedures were carried out according to the manufacturer's instructions.

2.2.4 Transmission electron microscopy

Bacteria were grown for 48 hours at 37°C and resuspended in 2% bovine serum albumin (BSA) in phosphate buffered saline (PBS). The bacteria were then incubated for 4 minutes on 200-mesh copper grids coated with formvar and stained in 2% tungstophosphoric acid (pH 6.8) for 2 minutes. Grids were examined using a Joel EM100SX-1 transmission electron microscope.

CHAPTER III

Outer Membrane Protein 87 (Omp87) Identification

Bioinformatics and Proteomics

3. Introduction

Legionella spp. continue to remain a health hazard worldwide, which are capable of causing debilitation and death in their unsuspecting hosts. As most of the victims of the disease are immunocompromised, there is an added urgency for the rapid diagnosis of the disease, so that the appropriate antibiotic treatment can be promptly administered.

Current detection methods are becoming increasingly efficient at diagnosing *Legionella* infections, however, outbreaks of the disease continue to occur, and the lives of the elderly and immunocompromised who succumb to this disease continue to remain at risk. Prevention of *Legionella* outbreaks, by continuous biomonitoring of water sources, or vaccination of 'at-risk' groups, would therefore provide a promising solution to this on-going problem.

The outer membrane provides bacterial cells with a semi-permeable and protective sheath, which surrounds the peptidoglycan capsule of the cell (Nikaido, 2003). The medical importance of outer membrane proteins has long been recognised. They are usually surface exposed molecules, which, for intact bacteria, makes them ideal target antigens for immune responses or detection systems (Palmer, 2002).

The identification of novel outer membrane proteins which can be used as therapeutic or diagnostic targets can often be a laborious and time consuming process, involving hours of laboratory-based experiments. Another more feasible approach, however, is the use of bioinformatics. Bioinformatics has become a powerful tool in the identification and analysis of DNA and proteins. Many computational programs are available today which perform a vast array of functions, such as sequence alignments, predictions of protein structures and function, and the determination of phylogenetic linkages. The development of highly complex statistical algorithms and computational models has drastically intensified their capacity to perform tasks which would once have taken days or weeks of calculations and analysis.

Many bioinformatics programs share databases of information which contain thousands of DNA and protein sequences. These databases are the key to predicting gene and protein

functions and structures, as novel sequences can be compared to these annotated and characterised genes and proteins to determine their likely significance, homology or function.

DNA sequence analysis is becoming an increasingly popular means for the identification of novel proteins, particularly when we consider that the DNA sequence of the genomes of many organisms, both prokaryotic and eukaryotic, are constantly being elucidated. Protein motifs, or domains, are short sequences of peptides which are unique to particular protein families, and can often provide a great deal of insight into the proteins structure and/or function. These motifs can be very useful for screening sequences, and for determining the family from which the novel protein or DNA sequence pertains.

Outer membrane proteins are one group of proteins, for which many motifs have been established, and have become publicly available through bioinformatics databases.

3.1 Outline of this chapter

The recently published *Legionella pneumophila* genome (Chien *et al*, 2004) will be screened for novel outer membrane proteins, through the use of bioinformatics programs. Any novel proteins found will then be analysed to determine which of these would possess potential for use as a diagnostic or vaccine target molecule.

Conventional laboratory methods will also be performed simultaneously, in an attempt to identify novel proteins through laboratory proteomics, such as Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and protein sequencing methods, such as matrix-assisted laser desorption/ionisation - time of flight mass spectrometry (MALDI-TOF MS). A comparison of methods used for outer membrane protein isolation from bacterial cells will also be performed.

3.2 Materials and Methods

3.2.1 Screening of the *Legionella pneumophila* genome - Bioinformatics

3.2.2 National Centre for Bioinformatics Information (NCBI)

The National Centre for Bioinformatics Information (NCBI), found at http://www.ncbi.nlm.nih.gov/ was used for screening the *Legionella pneumophila* genome. Protein motifs were searched for in this genome using the BLAST feature of the program.

3.2.2.1 *Ex*pert *P*rotein *A*nalysis *Sy*stem (ExPASy) and Prosite

The **Ex**pert **P**rotein **A**nalysis **Sy**stem (ExPASy) program, found at http://www.expasy.org/ was used for finding bacterial outer membrane protein motifs. In particular, the protein families and domains database, Prosite, was used. The following terms were entered into the database: 'bacterial outer membrane protein', 'membrane', 'bacterial surface antigen', and bacterial membrane protein'. A list of protein motifs was then obtained. These motifs were then used to screen the *L. pneumophila* serogroup 1 Philadelphia 1 strain genome, using the NCBI database.

3.2.2.2 European Bioinformatics Institute (EBI) : InterProScan and PFam

The PFam database of the European Bioinformatics Institute (EBI), found at http://www.ebi.ac.uk/ was used to obtain outer membrane protein families or domains. The sequence of a representative protein from each of these families was then used for screening of the *L. pneumophila* genome, in an attempt to find additional *L. pneumophila* outer membrane proteins. The 'Text Search' feature was used, and various searches were carried out using different search queries. The search terms 'surface antigen', 'outer membrane protein', 'surface exposed', 'surface antigen' and 'membrane' were used. When a match was obtained with the *L. pneumophila* genome, the *L. pneumophila* protein sequence was then used to perform a BLAST analysis against the NCBI microbial genome database.

3.2.3 Transmission Electron Microscopy studies of *L. pneumophila*

Transmission Electron Microscopy studies were performed on *L. pneumophila* AA100 cells (kindly provided by Prof. Yousef Abu Kwaik, from the University of Kentucky, Lexington, U.S.A). The method followed for the preparation of the TEM samples is detailed in Chapter 2, Materials and methods.

3.2.4 Outer membrane Protein Isolation – Comparison of methods

In all of the following methods, the strain used for the extraction of outer membrane proteins was *L. pneumophila* serogroup 1 AA100.

3.2.4.1 Ultracentrifugation

This method was based on that of Butler *et al* (1985) for bacterial outer membrane extraction. Briefly, freshly grown cells of *L. pneumophila* AA100 were suspended in 5 ml of 50 mM tris-HCl, pH 7.2, to an OD of 0.2. They were then centrifuged at 10,000 x *g* for 15 min to pellet cells. Membranes were then disrupted by adding 2% N-lauroyl sarcosinate (Sarkosyl) in 50 mM tris-HCl, pH 7.2. Cells were then incubated for 1 hr at 37°C, followed by sonication (6 cycles x 15 sec) in an ice bath. Lysozyme (1mg/ml) was then added, and the solution was again incubated at 37°C for 1 hr. Unbroken cells were then removed by low speed centrifugation. Outer membranes were then pelleted by ultracentrifugation at 100,000 x *g* for 30 min. They were then washed once in 2% Sarkosyl, and were finally suspended in solubilisation buffer, containing 2% SDS, 10% glycerol, 62.5 mM tris-HCl, 0.005% bromphenol blue, and 10% (vol/vol) 2-mercaptoethanol.

3.2.4.2 Sucrose density gradient

The protocol used was based on the method of Hindahl and Iglewski (1986) for the isolation of bacterial outer membranes. Briefly, 100 ml of overnight cultures of *L. pneumophila*

AA100 grown in BYE broth were harvested by centrifugation at 5000 x *g* for 15 min at 4°C. Cells were then washed twice in cold 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer, pH 7.4. DNase and RNase were added at a concentration of 50 µg/ml. The suspension was then sonicated on ice (6 x 15 sec) and whole cells were then removed by centrifugation at 1000 x *g* for 15 min. The supernatant was centrifuged at 250,000 x *g* for 1 hr. The pellet was then washed twice in cold HEPES buffer. The pellet was finally suspended in 1.5 ml of HEPES buffer, and 0.8 ml was loaded onto sucrose gradients consisting of 2 ml of 70% sucrose, 3 ml of 64% sucrose, 3 ml of 58% sucrose, 2 ml of 52% sucrose, and 1 ml of 46% sucrose. The gradient was then centrifuged at 63,000 x *g* at 4°C for 18 hr. The recovered membranes were washed twice in HEPES buffer by again centrifuging at 250,000 x *g* for 1 hr. Membranes were then resuspended in solubilisation buffer (as described above in previous method).

3.2.4.3 Sonication and Sarkosyl membrane disruption

This method was adapted from that of Crosa and Hodges (1981). Briefly, *L. pneumophila* AA100 cells grown on BCYE-α plates were harvested in 3 ml of 10mM Tris buffer, containing 0.3% (w/v) NaCl (pH 8.0). The sample was then sonicated 3 x for 30 sec, followed by a centrifugation step at 10,000 x *g* for 2 min. The supernatant was collected and subjected to a further centrifugation step at 17,000 x *g* at 4°C for 1 hour. The cell envelope was then incubated at room temperature with 3% Sarkosyl in 10mM Tris-HCl for 20 min. The outer membrane proteins were then collected by centrifugation at 17,000 x *g* for 1 hour.

3.2.4.4 Glycine-acid extraction

This method was based on the method of Garvis *et al* (1996). Freshly grown *L. pneumophila* AA100 cells were pelleted by centrifugation at 6,000 x *g* for 10 min at 4°C. They were then washed twice with 10mM phosphate buffered saline (PBS), pH 7.0. Cells were then suspended in 0.2M glycine-HCl, pH 2.2 and the suspension was stirred at room temperature for 2 hours. Bacterial cells were then removed by centrifugation at 6,000 x *g* for 20 min. The

supernatant was removed and neutralised with NaOH, and then concentrated using a Centricon-3 (Amicon, Inc., Beverly, Massachusetts, U.S.A.).

3.2.5 Outer membrane Protein Sequencing: Matrix Assisted Laser Desorption/Ionisation - Time of Flight Mass spectrometry (MALDI-TOF MS)

Outer membrane proteins were separated by SDS-PAGE on a maxi size gel. The protein bands of interest were then excised using a clean scalpel. The gel slices were then transferred to eppendorf tubes which were later sealed with parafilm. The samples were sent to the Australian Proteome Analysis Facility (Macquarie University, North Ryde, NSW) for MALDI-TOF mass spectrometry. Here, the samples were subjected to a tryptic digestion, and MALDI-TOF analysis was performed on the samples using an Applied Biosystems 4700 Proteomics Analyser with TOF/TOF optics in MS mode.

Results from the sequencing analysis were returned as a Peptide Mass Fingerprint (PMF), and in an MS Spectral output. The PMF data was then analysed using the Mascot feature of the MatrixScience program (www.MatrixScience.com; Matrix Science Ltd, London, U.K). The variable parameters for the analysis were used as indicated by the Australian Proteome Analysis Facility. These variable parameters used were as follows: 1) MS/MS Search; 2) Database: MSDB; 3) Taxonomy: Other proteobacteria; 4) Enzyme: Trypsin; 5) Max. missed cleavage: 1; 6) Variable modifications: Oxidation (M), Propionamide (C); 7) Monoisotopic; 8) Peptide tolerance: +/- 50ppm; 9) Peptide charge: 1+; 10) Data format: Mascot generic; 11) MS/MS tolerance: +/- 0.8Da; 12) Instrument: MALDI-TOF-TOF.

3.3 Results

3.3.1 Screening of the *L. pneumophila* genome: Bioinformatics

One hundred and twenty protein motifs were obtained from the searches of the Expasy (Prosite) bioinformatics program. These motifs are presented by the program as a consensus sequence, which is essentially the 'backbone' sequence of the motif. This sequence represents the order and combination of amino acids possible for any given motif. For each motif, there are therefore many possible combinations of amino acids which may be present

for different microbial species, within the same protein family. Based on the consensus sequence of the motif, all of the possible amino acids sequence combinations in the motif are determined by the Prosite program. All of these individual combinations were then used for the screening of the *L. pneumophila* genome.

A significant proportion of the screened motifs were found to possess similarity to the *L. pneumophila* genome. The proteins containing these motifs were then subjected to a BLAST analysis against the entire collection of proteins contained in the collective NBCI databases. The matches obtained with the highest similarity (i.e. the lowest E value) are shown in **Table 3.1**. The E value of the matches represents the likelihood that the match is occurring by a chance event. The closer this value is to zero (i.e. the smaller the value) the higher the likelihood that the match is not purely a chance event.

Table 3. 1 - Protein motif matches with the *L. pneumophila* genome. The table shows: column 1) the protein family name 2) the consensus sequence for that protein family 3) the actual protein motif which matched the *L. pneumophila* genome 4) the NCBI accession number for the matching protein in the *L. pneumophila* genome 5) the motif position within the *Legionella* protein 6) the name and size of the matching *Legionella* protein 7) NCBI BLAST matches with the microbial genome database 8) the E value for each BLAST result.

MOTIF NAME	CONSENSUS PATTERN	MATCHING MOTIF	NCBI ACCESSION NUMBER and PROTEIN SIZE	Position of Motif within Protein	BLAST RESULTS	PROTEIN NAME	E VALUE
5' nucleotidase_2 signature	[FYP]-x(4)-[LIVM]-G-N-H-E-F	FDVGTVGNHEFD	YP_096682.1 (575 aa)	134-143	*B. anthracis* *Ps. putida* *D. radiodurans*	5' nucleotidase	1 e^{-86} 6 e^{-53} 8 e^{-53}
AAA- Protein family signature	[LIVMT]-x-[LIVMT]-[LIVMF]-x-[GATMC]-[ST]-[NS]-x(4)-[LIVM]-D-X-A-[LIFA]-x -R	VIVIATNRPD- VLDPALTR	YP_096792.1 (639 aa)	292-313	*V. paraheamolyticus* *V. cholerae* *Shewanella oneidensis*	Cell Division protein (FtsH)	0.0 0.0 0.0

MOTIF NAME	CONSENSUS PATTERN	MATCHING MOTIF	NCBI ACCESSION NUMBER and PROTEIN SIZE	Position of Motif within Protein	BLAST RESULTS	PROTEIN NAME	E VALUE
ABC-2 Type Transporter	[LIMST]-x(2)-[LIMW]-x(2)-[LIMCA]-[GSTC]-x-[GSAIV]-x(6)-[LIMGA]-[PGSNQ]-x(9,12)-P-[LIMFT]-x-[HRSY]-x(5)-[RQ]	LTPLTYLGVVFY-SLTILPPFWQG	YP_094431.1 (257 aa)	179-200	*Ps. syringae pv. syringae* (B728a) *Ps. syringae pv tomato* str. (DC3000) *Ps. putida (*KT2440)	ABC Transporter, permease protein	3 e^{-76} 4 e^{-75} 3 e^{-74}
ATP synthase gamma subunit signature	[IV]-T-x-E-X(2)-[DE]-x(3)-G-A-x-[SAKR]	ITQELLEVVGGAEA	YP_096975.1 (288aa)	274-287	*Ps. aeruginosa* (PAO1) *Ps. putida* (KT2440) *Ps. syringae pv tomato str*. (DC3000)	ATP synthase gamma chain/subunit	1 e^{-105} 7 e^{-104} 1 e^{-102}

MOTIF NAME	CONSENSUS PATTERN	MATCHING MOTIF	NCBI ACCESSION NUMBER and PROTEIN SIZE	Position of Motif within Protein	BLAST RESULTS	PROTEIN NAME	E VALUE
ATP-binding cassette, ABC transporter-type, signature and profile	[LIVMFYC]-[SA]-[SAPGLVFYKQH]-G-[DENQMW]-[KRQASPCLIMFW]-KRNQSTAVM]-[KRACLVM]-[LIVMFYPAN]-{PHY}-[LIVMFW]-[SAGCLIVP]-{FYWHP}-{KRHP}-[LIVMFYWSTA]	LSGGQQQ- RVAIARAL	YP_096916.1	151-165	*S. pneumoniae* R6 *Nostoc sp.*	ABC transporter ATP-binding protein	4 e^{-128} 4 e^{-121}
ABC Transporter 1	[LIVMFYC]-[SA]-SAPGLVFYKQH]-G-[DENQMW]-[KRQASPCLIMFW]-[KRNQSTAVM]-[KRACLVM]-[LIVMFYPAN]-{PHY}-LIVMFW]-[SAGCLIVP]-{FYWHP}-{KRHP}-[LIVMFYWSTA]	VSAGDFALILGLSM	YP_095642	279-292	*S. mutans* (UA159) *V. parahaemolyticus (RIMD 2210633)* *Nostoc sp.*	ABC transporter, ATP binding protein	2 e^{-66} 5 e^{-63} 6 e^{-63}

MOTIF NAME	CONSENSUS PATTERN	MATCHING MOTIF	NCBI ACCESSION NUMBER and PROTEIN SIZE	Position of Motif within Protein	BLAST RESULTS	PROTEIN NAME	E VALUE
Alanine Dehydrogenase	[LIVM](2)-G-[GA]-G-x-A-G-x(2)-[SA]-(3)-[GA]-x-[SG]-[LIVM]-G-A-x-V-x(3)-D	IIGGGQAGTNAAK-IALGLGADVTILD	YP_094958.1 (373aa)	173-198	*V. cholerae*	Alanine Dehydrogenase	3 e^{-94}
Amino Acid Permeases Signature	[STAGC]-G-[PAG]-x(2,3)-[LIVMFYWA](2)-x-[LIVMFYW]-x-[LIVMFWSTAGC](2)-[STAGC]-x(3)-[LIVMFYWT]-x-[LIVMST]-x(3)-[LIVMCTA]-[GA]-E-x(5)-[PSAL]	AGPIGAIAYILGGFLM-YIVMLCLGELAVAMP	YP_095004.1 (487aa)	57-80	*B. Anthracis* *B. subtilis subsp. Subtilis* str. 168 *Ps. putida* KT 2440	Amino Acid Permease	4 e^{-81} 7 e^{-78} 2 e^{-73}

MOTIF NAME	CONSENSUS PATTERN	MATCHING MOTIF	NCBI ACCESSION NUMBER and PROTEIN SIZE	Position of Motif within Protein	BLAST RESULTS	PROTEIN NAME	E VALUE
Bacterial Export FHIPEP family signature	R-[LIVM]-[GSA]-E-V-[GSA]-A-R-F-[STAIV]-L-D-[GSA]-[LM]-P-G-K-Q-M-[GSA]-I-D-[GSA]-[DA]	RISEVSARFTLDA-MPGKQMAIDAD	YP_095811.1 (692aa)	141-164	*Ps. aeruginosa* (PAO1) *Ps. putida* (KT2440) *Ps. syringae pv. tomato str. (DC3000)* *Shewanella oneidensis MR-1*	Flagellar Biosynthesis Protein (FlhA)	0.0 0.0 0.0 0.0
Bacterial type II secretion system protein D signature	[GR]-[DEQKG]-[STVM]-[LIVMA](3)-[GA]-G-[LIVMFY]-X(11)-[LIVM]-P-[LIVMFYWGS]-[LIVMF]-[GSAE]-x-[LIVM]-P-[LIVMFYW](2)-x(2)-[LV]-F	GDIVVLGGLIQDSIG-NDNNKLPILGDIPGIGRLF	YP_095350.1 (791aa)	673-706	*Erwinia chrysanthemi* *B. pseudomallei*	Type II protein secretion LspD	$2\ e^{-51}$ $1\ e^{-50}$
Band 7 protein family signature	R-x(2)-[LIV]-[SAN]-x(6)-[LIV]-D-x(2)-T-x(2)-W-G-[LIVT]-	RDSINSRIIRIVDEA-TNPWGIKVTRIEIR	YP_096950.1 (259aa)	139-167	*Ralstonia solanacearum*	Stomatin-like protein	$1\ e^{-95}$

MOTIF NAME	CONSENSUS PATTERN	MATCHING MOTIF	NCBI ACCESSION NUMBER and PROTEIN SIZE	Position of Motif within Protein	BLAST RESULTS	PROTEIN NAME	E VALUE
	[KRH]-[LIV]-x-[KRA]-[LIV]-E-[LIV]-[KRQ]				*C. burnetti (RSA493)*		$3\ e^{-95}$
CO II and nitrous oxide reductase dinuclear copper centers signature (*Cytochrome c oxidase*)	V-x-H-x(33,40)-C-x(3)-C-x(3)-H-x(2)-M (The two C's and H's are copper ligands)	VHSWWVPEL-GVKRDAIPG	YP_096891.1 (401aa)	194-211	*Shewanella oneidensis* (MR-1) *Ps. aeruginosa* (PAO1) *V. parahaemolyticus* (RIMD2210663)	Cytochrome c Oxidase, subunit II	$9\ e^{-90}$ $5\ e^{-85}$ $9\ e^{-84}$
Cyclophilin-type peptidyl-prolyl cis-trans isomerase signature & profile	[FY]-x(2)-[STCNLV]-x-F-H-[RH]-LIVMN]-[LIVM]-x(2)-F-[LIVM]-x-Q-[AG]-G	YNGLTFHRVIAGFMIG	YP_095978.1 (188aa)	74-88	*Corynebacterium glutamicum* (ATCC 13032) *M. leprae* *Streptomyces chrysomallus*	Peptidyl-prolyl cis-trans isomerise	$2\ e^{-46}$ $6\ e^{-43}$ $3\ e^{-42}$
Cytochrome b/b6 signatures	[DENQ]-x(3)-G-[FYWMQ]-x-[LIVMF]-R-x(2)-H (H is a heme b562 ligand)	DINFGWLLRYMH	YP_096709.1 (404aa)	76-87	*Ps. putida* (KT2440) *Shewanella oneidensis* (MR-1) *V. cholerae*	Cytochrome c reductase	$1\ e^{-169}$ $2\ e^{-159}$ $3\ e^{-150}$

MOTIF NAME	CONSENSUS PATTERN	MATCHING MOTIF	NCBI ACCESSION NUMBER and PROTEIN SIZE	Position of Motif within Protein	BLAST RESULTS	PROTEIN NAME	E VALUE
D-isomer specific 2-hydroxyacid dehydrogenases signature	[LIVMFYWA]-[LIVFYWC]-x(2)-[SAC]-[DNQHR]-[IVFA]-[LIVF]-x-[LIVF]-[HNI]-x-P-x(4)-[STN]-x(2)-[LIVMF]-x-[GSDN]	VGTVGVGRIGERVI RRLKPFDCKELLYD	YP_094337.1 (403aa)	200-222	*Sinorhizobium meliloti* *Hyphomicrobium* sp. (JC17) *Pseudomonas sp.* (101)	NAD+ dependent formate dehydrogenase	1 e^{-166} 3 e^{-166} 2 e^{-163}
Dihydroorotate Dehydrogenase signature	[GS]-x(4)-[GK]-[GSTA]-[LIVFSTA]-[GST]-x(3)-[NQR]-x-G-[NHY]-x(2)-P-[RT]	FIEVGTVTDA-AQEGNPKPR	YP_095847.1 (388aa)	130-148	*H. influenzae* *V. vulnificus* (CMCP6) *V. cholerae*	Dihydroorotate Dehydrogenase	7 e^{-99} 3 e^{-97} 1 e^{-96}
FKBP-type peptidyl-prolyl cis-trans isomerase	[LIVMC]-x-[YF]-x-[GVL]-x(1,2)-[LFT]-x(2)-[G-x(3)-[DE]-[STAEQK]-[STAN]	LIDGTVFDST	YP_094827.1 (235aa)	157-166	*L. pneumophila* serogroup 8 *L. pneumophila* serogroup 1	Macrophage Infectivity Potentiator (MIP)	2 e^{-128} 8 e^{-128}
Ferrochelatase signature	[LIVMF](2)-x-[ST]-x-H-[GS]-[LIVM]-P-x(4,5)-[DENQKR]-x-G-[GP]-x(1,2)-Y	LLFSYHGIPER	YP_094469.1 (332aa)	183-193	*C. burnetii* *Ps. syringae pv. Syringae* (B728a) *Ps. syringae pv. Tomato str.* (DC3000)	Ferrochelatase	3 e^{-88} 9 e^{-68} 8 e^{-67}

MOTIF NAME	CONSENSUS PATTERN	MATCHING MOTIF	NCBI ACCESSION NUMBER and PROTEIN SIZE	Position of Motif within Protein	BLAST RESULTS	PROTEIN NAME	E VALUE
Flagellar motor protein MotA family signature	A-[LMF]-x-[GAT]-T-[LIVMF]-x-G-x-[LIVMF]-x(7)-P	AMVGTFLGILIAYGFISP	YP_096327.1 (301aa)	205-222	*S. enterica* subsp. *enterica* serovar *Typhi* *Y. pestis (CO92)*	Motility Protein A	2 e^{-87} 3 e^{-87}
Fumarate reductase/succinate dehydrogenase FAD-binding site	R-[ST]-H-[ST]-x(2)-A-x-G-G (H is the FAD binding site)	RSHTVAAQGG	YP_094573.1 (589aa)	43-52	*V. vulnificus* (CMCP6) *V. parahaemolyticus* (RIMD2210633) *S. typhimurium*	Succinate dehydrogenase	4 e^{-171} 3 e^{-170} 4 e^{-170}
Guanylate cyclases signature and profile	G-V-[LIVM]-x(0,1)-G-x(5)-[FY]-x-[LIVM]-[FYW]-[GS]-[DNTHKW]-[DNT]-[IV]-[DNTA]-x(5)-[DE]	VWSNDVTLAN	YP_095519.1 (441aa)	376-385	*Nostoc* sp. (PCC7120) *Leptospira interrogans serovar lai str. (56601)* *Anabaena* sp.	Adenylate cyclase	5 e^{-44} 6 e^{-40} 7 e^{-38}

MOTIF NAME	CONSENSUS PATTERN	MATCHING MOTIF	NCBI ACCESSION NUMBER and PROTEIN SIZE	Position of Motif within Protein	BLAST RESULTS	PROTEIN NAME	E VALUE
Guanylate Kinase signature and profile	T-[ST]-R-x(2)-[KR]-x(2)-[DE]-x(2)-G-x(2)-Y-x-[FY]-[LIVMK].	TTRPQRKQ- DANGEEYFFI	YP_096026.1 (209aa)	41-58	*C. burnetii* *Ps. aeruginosa (PAO1)* *Buchnera aphidicola*	Guanylate kinase	5 e^{-61} 2 e^{-56} 4 e^{-56}
Hexapeptide-repeat containing-transferase signature	[LIV]-[GAED]-x(2)-[STAV]-x-[LIV]-x(3)-[LIVAC]-x-[LIV]-[GAED]-[STAVR]-x-[LIV]-[GAED]-x(2)-[STAV]-x-[LIV]-x(3)-[LIV]	IGSGAMIMPGIKIG- HGAVIGSRALVAKDV	YP_094790.1 (202aa)	162-190	*Bacteroides fragilis* *Oceanobacillus iheyensis* HTE831	Acetyltransferase (Putative)	1 e^{-28} 8 e^{-26}
HlyD family secretion proteins signature	[LIVM]-x(2)-G-[LM]-x(3)-[STGAV]-x-[LIVMT]-x-[LIVMT]-[GE]-[KR]-x-[LIVMFYW](2)-x-[LIVMFYW](3)	IRPGMAVQVFV- RTGERSLLNYL	YP_095546.1 (378aa)	342-363	*Ps. putida* (KT2440) *V. parahaemlolyticus* *Shewanella oneidensis* MR-1	HlyD family secretion protein	2 e^{-76} 3 e^{-62} 6 e^{-62}
MraY family signatures (Phospho-N-acetylmuramoyl-pentapeptide-transferase)	[KRA]-x(2)-[TIVK]-P-[ST]-[MGA]-[GA]-G-[LIVSA]-x-[LIVMF](2)	KKYTPTMGGLVIL	YP_096622.1 (372aa)	79-91	*C. burnetii* (RSA 493) *Ps. putida* KT2440 *Ps. aeruginosa*	Phospho-N-acetylmuramoyl-pentapeptide-transferase	8 e^{-145} 7 e^{-138} 6 e^{-137}

MOTIF NAME	CONSENSUS PATTERN	MATCHING MOTIF	NCBI ACCESSION NUMBER and PROTEIN SIZE	Position of Motif within Protein	BLAST RESULTS	PROTEIN NAME	E VALUE
PEP- utilizing enzymes signatures	G-[GA]-X-[stn]-x-[STN]-x-H-[STA]-[STAV]-[LIVM](2)-[STAV]-[RG] (H is phosphorylated)	GGKTSHAAVVAR	YP_094840.1 (795aa)	417-428	*Ps. aueruginosa* (PAO1) *Ps. putida* (KT2440) *E. coli* 0157:H7	Phosphoenolpyruvate Synthase	2 e^{-137} 3 e^{-136} 1 e^{-135}
PTR2 family proton/oligopeptide symporters signatures	[GA]-[GAS]-[LIVMFYWA]-[LIVM]-[GAS]-D-x-[LIVMFYWT]-[LIVMFYW]-G-x(3)-[TAV]-[IV]-x(3)-[GSTAV]-x-[LIVMF]-x(3)-[GA]	GGWVADRLLGAS-RTIFIGGILITIG	YP_095407.1 (480aa)	62-74	*V. cholerae*	Proton/peptide symporter family protein	9 e^{-112}
PTS HPr component phosphorylation sites signature	[GSTADE]-[KREQSTIV]-x(4)-[KRDN]-S-[LIVMF](2)-x-[LIVM]-x(2)-[LIVM]-[GADE] [S is phosphorylated]	GRQVDAKSIMGVMMLA	YP_094519.1 (89aa)	42-56	*N. meningitidis* (MC58) *Ralstonia eutropha* *Nitrosomonas europaea* (ATCC 19718)	Phosphocarrier protein HPr	7 e^{-22} 1 e^{-19} 1 e^{-17}

MOTIF NAME	CONSENSUS PATTERN	MATCHING MOTIF	NCBI ACCESSION NUMBER and PROTEIN SIZE	Position of Motif within Protein	BLAST RESULTS	PROTEIN NAME	E VALUE
Phosphatidate cytidylytransferase signature	S-x-[LIVMF]-K-R-x(4)-K-D-x-[GSA]-x(2)-[LIF]-[PG]-x-H-G-G-[LIVMF]-x-D-R-[LIVMFT]-D	SGFKRAFKIKDFG DSIPGHGGITDRMD	YP_094548.1 (284aa)	241-264	*C. burnetii* (RSA 493) *Ps. syringae pv. Tomato str.*	Phosphatidate cytidylyltransferase	4 e^{-40} 2 e^{-38}
Polyprenyl synthetase signatures	[LIVM](2)-x-D-D-x(2,4)-D-x(4)-R-R-[GH].	LIHDIPAMDNDSYRRG	YP_096339.1 (309aa)	91-107	*Xylella fastidiosa* Temecula 1 *V. cholerae* *Xylella fastidiosa*	Geranyltranferase	5 e^{-51} 7 e-50 2e^{-49}
Prolipoprotein diacylglyceryl transferase	G-R-x-[GA]-N-F-[LIVMF]-N-x-E-x(2)-G	GRIGNFINSEIWG	YP_096863.1 (256aa)	138-150	*C. burnetii* RSA493 *Ps. aeruginosa* (PAO1) *Ps. putida* (KT2440)	Prolipoprotein diacylglyceryl transferase	6 e^{-98} 7 e^{-94} 2 e^{-88}
Protein SecA signatures	[IV]-x-[IV]-[SA]-T-[NQ]-M-A-G-R-G-x-D-I-x-L	VTVATNMAGRGTDIVL	YP_095492.1 (902aa)	501-516	*Shewanella oneidensis* (MR-1) *E. coli 0157:H7* *S. enterica subsp. enterica serovar Typhi*	Preprotein translocase sec A subunit	0.0 0.0 0.0

MOTIF NAME	CONSENSUS PATTERN	MATCHING MOTIF	NCBI ACCESSION NUMBER and PROTEIN SIZE	Position of Motif within Protein	BLAST RESULTS	PROTEIN NAME	E VALUE
Protein SecY signatures	[GST]-[LIVMF]-[LIVMFCA]-x-[LIVMF]-[GSA]-[LIVM]-x-P-[LIVMFY](2)-x-[AS]-[GSTQ]-[LIVMFAT](3)-[EQ]-[LIVMFA](2)	SILALGIMPYISASIVVQLL	YP_094393.1 (444aa)	77-95	*C. burnetii* (RSA 493) *Shewanella oneidensis* (MR-1) *V. parahaemolyticus* (RIMD 2210663)	Preprotein translocase sec Y subunit	1 e^{-168} 7 e^{-159} 2 $^{e-155}$
Respiratory-chain NADH dehydrogenase 20 Kd subunit	[GN]-x-D-[KRHST]-[LIVMF](2)-P-[IV]-D-[LIVMF4YW](2)-x-P-x-C-P-S-[PT] The C is a putative 4Fe-4S ligand)	GTDKIVPVDVYIPGCPP	YP_096785.1 (158aa)	118-134	*Nitrosomonas europaea* ATCC 19718	Respiratory-chain NADH dehydrogenase 20 Kd subunit	9 e^{-75}
Respiratory-chain NADH dehydrogenase 30Kd subunit	E-R-E-x(2)-[DE]-[LIVMFY](2)-x(6)-[HK]-x(3)-[KRP]-x-[LIVM]-[LIVMYS]	EREVFDLFGILF SGHSDIRRIL	YP_096784.1 (227aa)	143-164	*C. burnetii* (RSA 493)	NADH dehydrogenase	3 e^{-129}
Respiratory-chain NADH dehydrogenase 49 Kd subunit	[LIVMH]-H-[RT]-[GA]-x-E-K-[LIVMTN]-x-E-x-[KRQ]	LHRETEKLIEHK	YP_096783.1 (422aa)	48-59	*C. burnetii* (RSA 493) *X. axonopodis pv. Citri* str. 306 *Xylella fastdiosa*	NADH dehydrogenase subunit D	0.0 9 e^{-177} 1 $^{e-175}$

MOTIF NAME	CONSENSUS PATTERN	MATCHING MOTIF	NCBI ACCESSION NUMBER and PROTEIN SIZE	Position of Motif within Protein	BLAST RESULTS	PROTEIN NAME	E VALUE
Respiratory-chain NADH dehydrogenase 51 Kd subunit	G-[AM]-G-[AR]-Y-[LIVM]-C-G-[DE](2)-[STA](2)-[LIM](2)-[EN]-S	GAGAYICGDESALIES	YP_096781.1 (428aa)	171-186	*C. burnetii* (RSA 493) *N. meningitidis* (MC58) *N. meningitidis (z2491)*	NADH dehydrogenase subunit F	2 e^{-174} 1 e^{-159} 2 e^{-158}
Respiratory-chain NADH dehydrogenase 75 Kd subunit	P-x(2)-C-[YWSD]-x(7)-G-x-C-R-x-C	PRFCYHERISIAGNCRMC	YP_096780.1 (783aa)	31-48	*C. burnetii* (RSA 493) *X. axonopodis pv. Citri* str. 306 *X. campestris pv. Campestris str.* ATCC 33913	NADH dehydrogenase subunit G	2 e^{-135} 1 e^{-116} 3 $^{e-116}$
SLC 26 a Protein family signature	[PAV]-x-[FY]-[GS]-L-Y-[STAG](2)-x(4)-[LIVFYA]-[LIVMST]-[YI]-x(3)-[GA]-[GST]-S-[KR]	PVYGLYASFFPAIIYLFFGTSR	YP_094633.1 (733aa)	365-380	*Bradyrhizobium japonicum* (USDA 110) *Mesorhizobium loti* *Rickettsia conorii*	2-Acylglycerophos-Phoethanolamine	2 e^{-144} 7 e^{-144} 6 e^{-143}
Signal peptidases II signature	[LIVM]-x-[GASF]-[GA]-[GAS]-[LIVMT]-[GAS]-N-[LVMFG]-[LIVFYG]-D-[RI]-[LIVMFA]	IGGGALGNLYDRL	YP_094972.1 (121aa)	67-76	*C. burnetti* *Y. pestis* (CO92) *E. coli* k12	Lipoprotein signal peptidase	1 e^{-39} 2 e^{-33} 6 e^{-33}

MOTIF NAME	CONSENSUS PATTERN	MATCHING MOTIF	NCBI ACCESSION NUMBER and PROTEIN SIZE	Position of Motif within Protein	BLAST RESULTS	PROTEIN NAME	E VALUE
Sodium dicarboxylate transporter	P-x(0,1) -G-[DE]-x-[LIVMF](2)-x-[LIVM](2)-[KREG]-[LIVM](3)	PIGATVNMDGAA-LFQCVAAVFIAQ	YP_096257.1 (430aa)	295-318	*Y. pestis* *Xanthomonas campestris pv. Campestris str. ATCC 33913* *Ps. aeruginosa*	C4- dicarboxylate transport protein	$2\ e^{-108}$ $3\ e^{-108}$ $2\ e^{-107}$
Sodium sulfate symporter	[STACP]-S-x(2)-F-x(2)-P-[LIVM]-[GSA]-x(3)-N-x-[LIVM]-V	ASSDFSTPIGYQTNlMV	YP_095305.1 (94aa)	42-58	*Brucella suis*	Transporter, TrkA family	$6\ e^{-25}$
Squalene and pytoene synthase (Also called Farnesyl-diphosphate farnesyltransferase)	Y-[CSAM]-x(2)-[VSG]-A-[GSA]-[LIVMAT]-[IV]-G-x(2)-[LMSC]-x(2)-[LIV]	YCYHVAGV-VGLMMARV	YP_094165.1	155-170	*N. europaea* ATCC 19718 *Ustilago maydis* *Halobacterium sp. (NRC-1)*	Squalene and pytoene synthase (366aa)	$3\ e^{-15}$ $4\ e^{-13}$ $4\ e^{-11}$
Succinate Deydrogenase		HTWNGIRHLMWDlG	YP_094571.1	86-99	*Ps. aeruginosa* *Ps. putida* KT2440	Succinate deydrogenase Cytochrome b556 (130aa)	$5\ e^{-32}$ $3\ e^{-31}$

MOTIF NAME	CONSENSUS PATTERN	MATCHING MOTIF	NCBI ACCESSION NUMBER and PROTEIN SIZE	Position of Motif within Protein	BLAST RESULTS	PROTEIN NAME	E VALUE
	R-P-[LIVMT]-x(3)-[LIVM]-x(6)-[LIVMWPK]-x(4)-S-x(2)-H-R-x-[ST]				*Ps. syringae pv. Tomato str.* DC3000		1 e^{-29}
Sugar transport proteins signature	[LIVMSTAG]-[LIVMFSAG]-x(2)-[LIVMSA]-[DE]-x-[LIVMFYWA]-G-R-[RK]-x(4,6)-[GSTA]	SGFLSDRFG-RRKILMTAA	YP_094583.1	282-296	*C. burnetti* (RSA 493)	Major facilitator family transporter (427aa)	6 e^{-97}
Tyrosine specific protein phosphatases signature and profile	[LIVMF]-H-C-x(2)-G-x(3)-[STC]-[STAGP]-x-[LIVMFY]	IHCLGGVGRTGTMAA	YP_096814.1 (319aa)	229-243	*Clostridium acetobutylicum*	Protein tyrosine phosphatases II superfamily	4 e^{-29}
ADH Zinc	G-H-E-x(2)-G-x(5)-[GA]-x(2)-[IVSAC]	GHEGAGSVVQIGKNV	YP_094738.1 (342aa)	64-78	*V. vulnificus* *V. parahaem.* (RIMD 2210633) *V. cholerae*	Threonine 3-deydrogenase	6 e^{-148} 1 e^{-147} 2 e^{-147}

MOTIF NAME	CONSENSUS PATTERN	MATCHING MOTIF	NCBI ACCESSION NUMBER and PROTEIN SIZE	Position of Motif within Protein	BLAST RESULTS	PROTEIN NAME	E VALUE
DnaJ domains signature and profile	[FY]-x(2)-[LIVMA]-x(3)-[FYWHNT]-[DENQSA]-x-L-x-[DN]-x(3)-[K]	FGAVGEAYQVLS DPGLRSKY	YP_096275.1 (296aa)	45-64	*N. europaea* (ATCC 19718) *C. burnetti* RSA 493	DnaJ/DNA binding protein	$5\ e^{-80}$ $5\ e^{-80}$

An additional search was also performed with the protein families database (PFam) within the European Bioinformatics Institute (EBI) database. This database does not perform protein motif searches, but searches for protein families and domains within a sequence.

A search was performed with the PFam program for protein families or domains using search parameters such as ‘membrane’, ‘outer membrane protein’, ‘surface exposed’ and ‘surface antigen’. This resulted in several protein family matches, along with bacterial species which possessed ‘representative’ proteins from each family. In order to then screen the *L. pneumophila* genome, one of the representative proteins was selected, and the sequence of the protein was used to perform an NCBI BLAST analysis with the *L. pneumophila* genome. When the BLAST analysis revealed a match with the *L. pneumophila* genome, the corresponding *L. pneumophila* sequence was then used for a BLAST analysis with the entire NCBI protein database. The results of the analysis are shown in **Table 3.2**.

Table 3.2 PFam protein family matches with the *Legionella* database. The table shows: column 1) the protein family name 2) The PFam accession number for the protein family 3) the name of the matching protein in the *Legionella* database and the NCBI accession number 4) the NCBI BLAST results with the microbial genome database 5) the E values of each BLAST result.

PROTEIN FAMILY NAME	PFam ACCESSION NO.	NCBI ACCESSION NO. and *L. pneumophila* PROTEIN NAME	BLAST RESULTS	PROTEIN NAME	E VALUE
Surface Antigen msp4 (OmpA of *E. coli*)	IPR002566	YP_094693.1 Outer Membrane Protein, OmpA family protein (249 aa)	*Pseudomonas fluorescens* (Pf-5) *Fusobacterium nucleatum subsp. vincentii* (ATCC 49256)	OmpA family protein Outer membrane protein	$9e^{-18}$ $5e^{-17}$
Bacterial surface antigen family (D15) (*H. influenzae*)	IPR000184	YP_094550.1 Hypothetical protein (786aa)	*Nitrosococcus oceani* (ATCC 19707)	Outer membrane protein	$3e^{-172}$

			(*Methylococcus capsulatus str. Bath*) (*Methylobacillus flagellatus* KT)	outer membrane protein, OMP85 family surface antigen (D15)	$5e^{-156}$ $2e^{-151}$
Virulence-related outer membrane protein family (OmpX of *E. coli*)	IPR000758	NC_002942.5 Hypothetical protein (276aa)	No significant Matches*		
Outer membrane efflux protein family (*E. coli*)	IPR003423	NC_002942.5 Hypothetical protein (67aa)	No significant Matches*		

* Matches were considered 'significant' when their E value was less than $1e^{-3}$

3.3.2 Transmission Electron Microscopy studies of *L. pneumophila*

Gram stains performed on cultures of *L. pneumophila* AA100 which had been passaged several times on BCYE-α agar showed normal sized *L. pneumophila* cells, that ranged between 2-20 μm in length. In addition to these cells however, long filamentous forms of *L. pneumophila* were seen.

TEM analysis of the *L. pneumophila* cells revealed long filamentous forms of the organism. This image is shown in **Figure 3.1.**

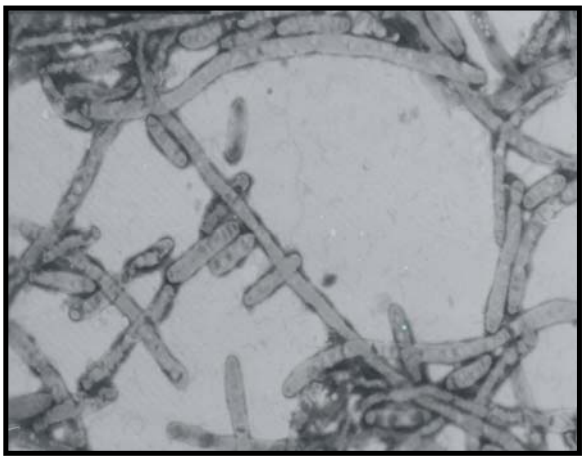


Figure 3.1. Transmission electron microscopy image of *L. pneumophila* AA100 showing long filamentous forms of the organism (x 6,000).

3.3.3 Outer membrane Protein Isolation – Comparison of methods

Four methods for the isolation of outer membrane proteins were compared, and the resulting samples were analysed by separating the protein preparations by SDS-PAGE. The SDS-PAGE image of the proteins extracted by each method is shown in **Figure 3.2**.

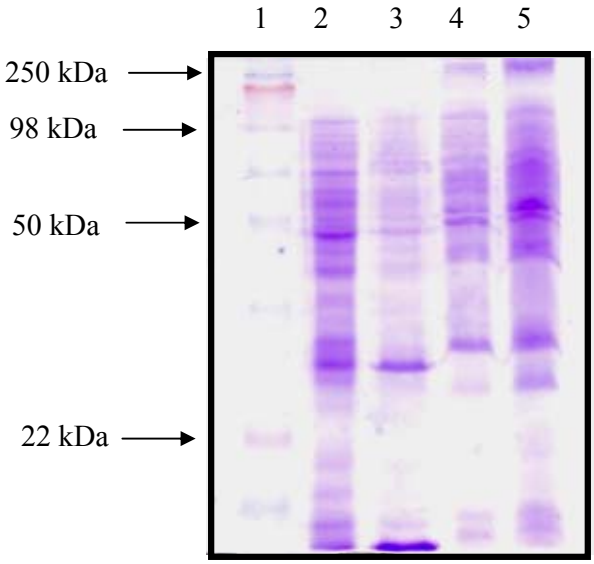


Figure 3.2. Comparison of four methods for the isolation of outer membrane proteins. Lane 1: SeeBlue® molecular weight marker; Lane 2: Omp isolation method 1 (Sonication and sarkosyl membrane disruption); Lane 3: Omp isolation method 2 (Sucrose density gradient); Lane 4: Omp isolation method 3 (Ultracentrifugation); Lane 5: Omp isolation method 4 (Glycine-acid extraction).

Of the four methods, the Glycine-acid extraction method was the simplest and easiest procedure to perform. It was performed within 3 hours, and required the least labour. This method also gave a clear profile of the isolated outer membrane proteins, which allowed for easy excision of the protein bands for mass spectrometry analysis. Therefore this method was chosen to further analyse the outer membrane proteins of *L. pneumophila*.

3.3.4 Outer membrane Protein Sequencing: MALDI-TOF Mass spectrometry

Numerous outer membrane proteins of *L. pneumophila* were selected for MALDI-TOF Mass spectrometry, in order to identify novel and previously uncharacterised proteins in *L. pneumophila*.

Glycine-acid extraction was used for the isolation of *L. pneumophila* outer membrane proteins. The isolated proteins were then separated by SDS-PAGE on a maxi size gel. The gel image is shown in **Figure 3.3**. The protein bands of interest were then excised and sequenced by MALDI-TOF Mass spectrometry. Five protein samples were sequenced, and the Peptide Mass Fingerprints (PMF) spectral images were obtained. An example of one of these spectral image results is shown in **Figure 3.4**. The PMF data was then analysed and the results of the analysis are shown in **Table 3.3.**

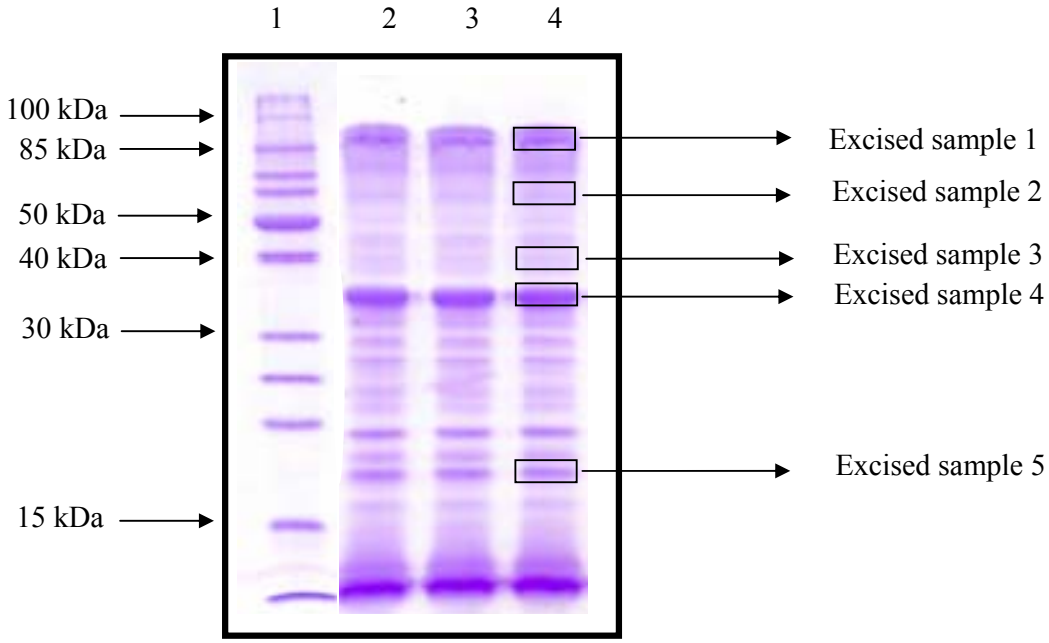


Figure 3.3. *L. pneumophila* outer membrane proteins separated by SDS-PAGE for MALDI-TOF Mass Spectrometry analysis. The *L. pneumophila* Omp samples were electrophoresed in triplicate. The boxes indicate which bands were excised and sequenced. Lane 1: Molecular weight standard (Fermentas Life Sciences, U.S.A); Lanes 2-4: *L. pneumophila* isolated outer membrane proteins (in triplicate).

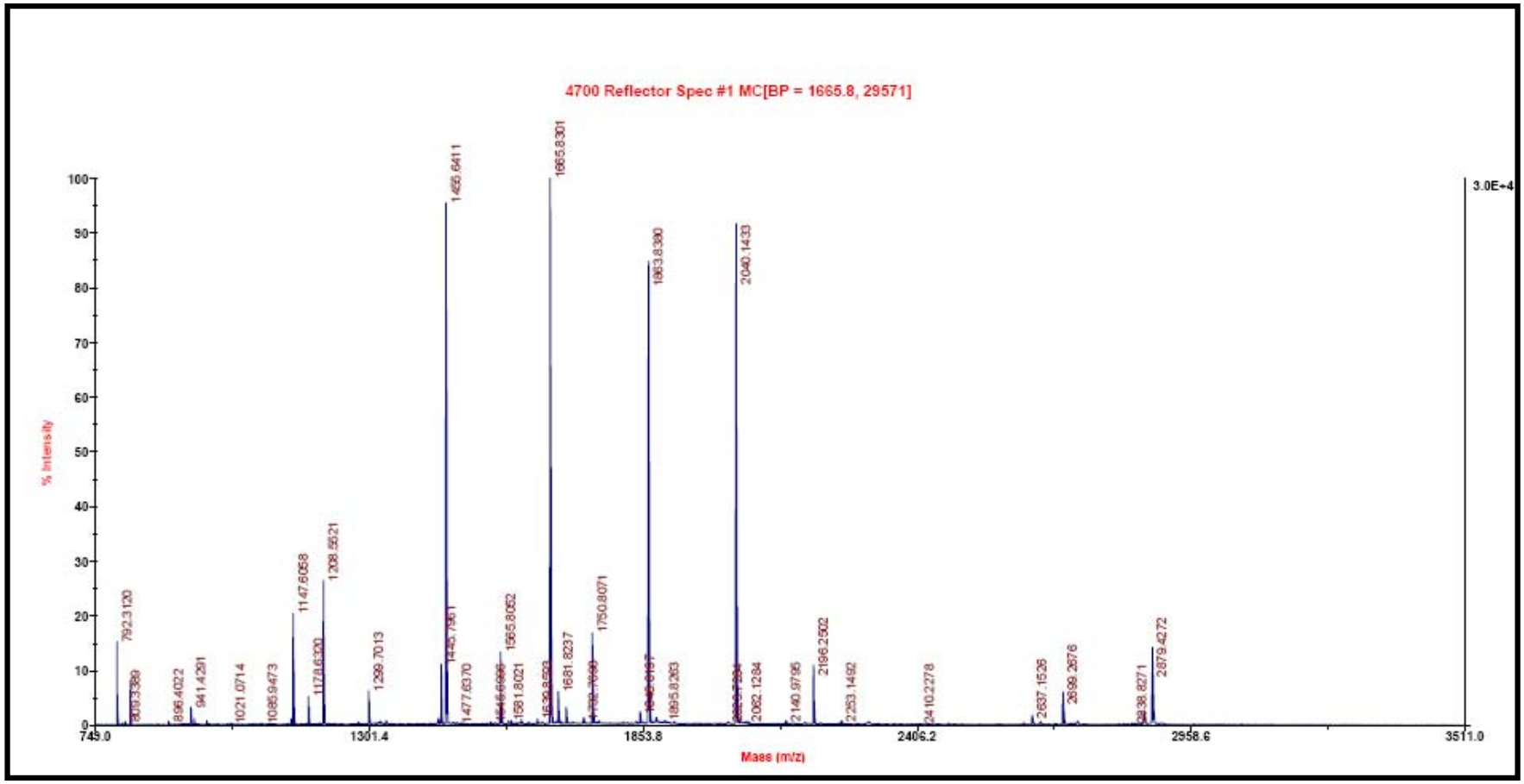


Figure 3.4. Spectral image of a Peptide Mass Fingerprint (PMF) result following MALDI-TOF sequencing of an *L. pneumophila* outer membrane protein.

Table 3.3. MALDI-TOF Mass spectrometry sequencing results of excised *L. pneumophila* outer membrane proteins. The columns show the excised protein sample number (Figure 3.2); the matching protein NCBI accession number; the name and size of the matching protein; the organism in which the protein is found; and the score of the match*.

PROTEIN SAMPLE No.	NCBI ACCESSION NO.	PROTEIN NAME and SIZE	ORGANISM	SCORE*
Sample 1	Q8VQ24_BARHE	Omp89 (89 kDa)	*Bartonella henselae*	68
Sample 2	CH60_LEGPA	60 kDa chaperonin Heat Shock Protein (58 kDa)	*Legionella pneumophila*	70
Sample 3	Q8PNP2_XANAC	outer membrane protein (39 kDa)	*Xanthomonas axonopodis pv. citri*	241

Sample 4	Q2WG57_LEGPN	Major outer membrane protein (32 kDa)	*Legionella pneumophila*	67
Sample 5	No Significant Match			

* The protein score is -10*Log(P), where P is the probability that the observed match is a random event. Protein scores of greater than 67 are significant ($p<0.05$) (www.MatrixScience.com).

3.3.5 Selection of outer membrane protein for further characterisation

From these search results, a protein was selected for further analysis and characterisation. The protein chosen belongs to the Omp85 bacterial surface antigen family. This protein corresponded to sample number 1 from the MALDI-TOF Mass spectrometry analysis, which revealed a match with an outer membrane protein (Omp89) from *Bartonella henselae*. Similarly, the protein was identified through the PFam bioinformatics analysis as an outer membrane protein belonging to the Omp85 family of proteins. The protein, which belongs to the Omp85 family of bacterial surface antigens, was selected for three reasons. Firstly, this protein was found in *L. pneumophila* using both bioinformatics, and the isolation of the protein through laboratory techniques, with subsequent MALDI-TOF mass spectrometry sequencing. Secondly, this protein has not yet been described in *Legionella*. Analysing this protein may therefore provide further insight into the mechanisms of *L. pneumophila* pathogenesis. Thirdly, this family of proteins is present on the bacterial outer membrane and is surface exposed. This criterion is important for the development of bacterial detection systems which usually rely on surface antigens for the detection of the organism.

3.3.6 BLAST Sequence analysis of the *L. pneumophila* genome with the *Haemophilus influenzae* D15 protein.

In order to determine the location of the novel protein in the *L. pneumophila* genome, a BLAST analysis was performed with the D15 Outer membrane antigen of *Haemophilus influenzae,* and the *L. pneumophila* genome.

The D15 Outer membrane antigen of *Haemophilus influenzae* is one representative of the Omp85 family of proteins which has been thoroughly characterised, and for which the protein sequence has been published (Flack *et al*, 1995).

The gene was found to be located at nucleotide positions 547,578-549,939 in the *L. pneumophila* genome. A sequence alignment was then performed, using the D15 protein sequence of *H. influenzae,* with the protein sequence of the novel *L. pneumophila* protein. This alignment is shown in **Figure 3.5.**

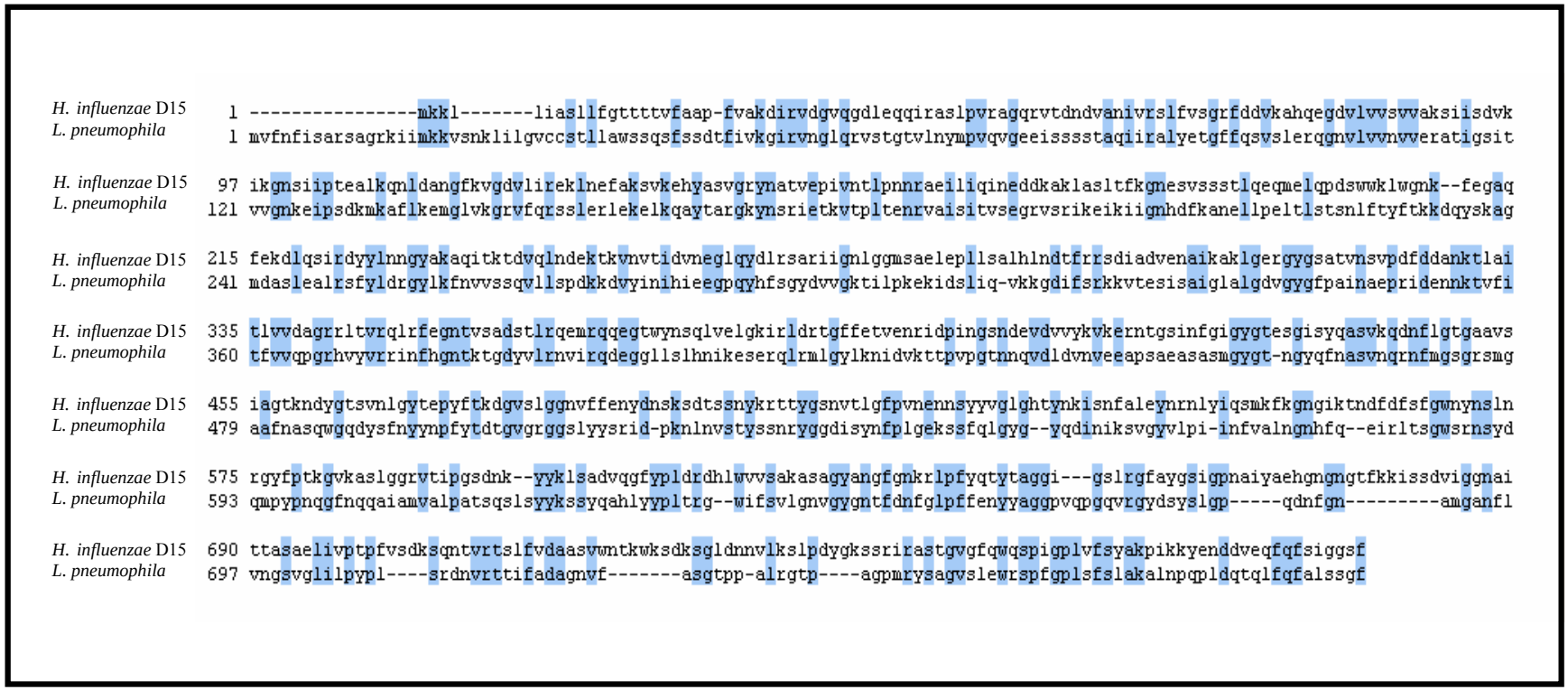


Figure 3.5. Sequence alignment of the *H. influenzae* D15 protein (NCBI accession no. AAC22575) with the *Legionella pneumophila* Philadelphia 1 genome (NCBI accession no NC_002942.5). The sequence produced an amino acid identity match of 29% (231/783) with an E value of $1e^{-105}$.

3.4 Discussion

Bioinformatics programs and databases are today considered an invaluable tool in many scientific research areas. In the field of bacterial biotechnology, bioinformatics is assisting researchers in areas as vast as the development of novel drugs and antimicrobial agents, the determination of protein biomarkers for various bacterial diseases, and the development of vaccines which are more effective and easier to administer (Bansal, 2005).

Program features are being continually updated and improved and programs are becoming increasingly specialised. A program was recently developed, for example, which can be used to identify unique bacterial strain, species, and genus-specific proteins (Mazumber *et al*, 2005).

The concept of using both proteomics and bioinformatics for the identification of novel proteins is one which also appears to be gaining prevalence. Berven *et al* (2006) recently utilised both conventional proteomics, such as 2-D gel electrophoresis and mass spectrometry, in conjunction with a novel biocomputing program, to analyse the outer membrane subproteome of *Methylococcus capsulatus.*

The bioinformatics analyses performed in this study, using the motifs found in outer membrane proteins, resulted in numerous matches with the *L. pneumophila* genome. The Expasy (Prosite) database was searched using key words such as 'membrane', 'outer membrane protein' and 'surface antigen', and the resulting list of protein motif sequences was used to perform an NCBI BLAST analysis with the *L. pneumophila* Philadelphia 1 genome. Although there were numerous matches, some of these proteins were not confined to the outer membrane of the cell, and included various enzymes and transporter proteins which may intermittently function in the bacterial outer membrane, but are not necessarily located within the outer membrane of the cell.

The protein families database (PFam), which is part of the European Bioinformatics Institute (EBI) was also used, and resulted in a few more matches with the *L. pneumophila* genome. These matches corresponded not to protein motif matches, but protein family matches. These protein family signatures are more complex than the protein motifs found using the Expasy database. The protein family or domain signatures are usually an entire set of motifs combined to cover and include all of the protein members of a family. Therefore, instead of a single signature or motif sequence,

a protein family or domain may consist of hundreds of different motifs strung together (http://www.ebi.ac.uk/).

When a protein family match was obtained with the *L. pneumophila* genome using the PFam database, a bacterial species possessing a representative of this protein was selected so that a protein BLAST analysis could be performed. In the case of the surface antigen msp4 family, for example, the OmpA of *E. coli* was selected, and the sequence of this protein was used for an NCBI BLAST analysis against the *L. pneumophila* genome (**Table 3.2)**. The *L. pneumophila* protein sequence was then used to perform an NCBI BLAST against the microbial genome database. This analysis revealed matches with the protein in organisms such as *Pseudomonas fluorescens* Pf-5, and *Fusobacterium nucleatum subsp. Vincentii.*

Transmission electron microscopy studies of *L. pneumophila* AA100 cells which had been passaged several times on BCYE-α agar, revealed that in addition to the expected sized cells (of around 2-20 μm in length), there were also long filamentous forms of the organism. This observation was first reported shortly after the discovery of the organism (Faine *et al*, 1979). Although the reasons for the development of these filamentous forms of the organism are not yet known, studies have indicated that the shorter bacilli are more virulent than the longer forms (Nowicki *et al*, 1987).

In addition to the bioinformatics approach, proteomic techniques were also used to identify novel outer membrane proteins. Four methods were compared for the isolation of outer membrane proteins. Methods 1 and 3 (2.2.1 and 2.2.3) were similar, and both involved the use of sonication and sarkosyl for the disruption of the bacterial membrane. Method 2 (2.2.2) involved a sucrose gradient for the separation of membrane components. This method proved to be laborious, and the layers of separated proteins were quite difficult to extract from the sucrose layered column. This method also contained an 18 hour ultracentrifugation step which made the entire process quite lengthy. Method 4 (2.2.4) utilised glycine-acid for the 'stripping' of the membrane proteins from the cell. This method was distinct from the others as it did not utilise detergents or lengthy centrifugation steps, and was the easiest to perform. The clarity and yield of the isolated proteins was comparable to the other methods, but the method was quicker to carry out.

The glycine-acid method was therefore selected as the method used for separating *L. pneumophila* outer membrane proteins for MALDI-TOF Mass spectrometry. The proteins were separated on a maxi gel which enabled for better resolution of the proteins and facilitated their excision from the gel. Numerous proteins were selected and excised from the gel. The proteins were then sequenced by MALDI-TOF Mass spectrometry and the Peptide Mass Fingerprints (PMF) results for each protein sample were further analysed using the Mascot feature of the Matrix Science program. This program enabled the identification of unknown proteins through the analysis of PMF samples against a database of typical PMF patterns.

Two of the proteins sequenced revealed matches with the *L. pneumophila* database. Only the smallest protein band excised, sample 5, did not display any relevant matches with the database. The largest protein (sample 1) revealed a match with the Omp89 protein of the related organism *Bartonella henselae*. Although this sample did not produce a match with the *L. pneumophila* database, the result was consistent with the protein size expected from the gel (~89 kDa) to the matching protein in *B. henselae*. This match also corresponded with the bioinformatics results, which indicated the presence in *L. pneumophila* of an outer membrane protein belonging to the Omp85 family of proteins. As this protein is novel in *L. pneumophila*, and has not yet been characterised, it would probably not contain a recognised PMF pattern in the database, meaning that a link with the protein in *L. pneumophila* would not be established during a PMF search.

Sample number three also did not produce a match with the *L. pneumophila* database, but instead revealed a match with a 39 kDa outer membrane protein of the closely related *Xanthomonas axonopodis.* This size again corresponds well with the size of the protein in the SDS-PAGE gel analysis, therefore indicating that this protein may also not yet have been characterised in *L. pneumophila*.

The other protein samples excised for MALDI-TOF analysis seemed to match well with the database. The sizes expected from the gel corresponded well with the sizes of protein matches observed in *L. pneumophila*. One difference however, albeit slight, was the result for the major outer membrane protein (mOMP), which was reported as being 32 kDa in size. The literature however, indicates that this protein is between 24-29 kDa, depending on the method used for its isolation (Engleberg *et al*, 1984).

A possible explanation for this slight difference may be that the protein is reported as often remaining linked with peptidoglycan upon isolation, even following treatment of

the cells with detergents such as SDS (Gabay and Horwitz, 1985). This may account for the minor increase in size observed. Gabay and Horwitz (1985) also reported the mOMP as being the most abundant protein in *L. pneumophila*. This is evident from the gel in Figure 3.3, which clearly shows that this protein band has a much greater intensity than any of the other proteins. Although the gel contains only outer membrane proteins, and does not represent the entire cell protein content, it is evident that this protein is by far the most abundant of the outer membrane proteins isolated.

Through the combination of bioinformatics, SDS-PAGE and MALDI-TOF mass spectrometry, a previously uncharacterised outer membrane protein was identified in *L. pneumophila*. This protein belongs to the Omp85 family of proteins, and includes proteins such as the *Haemophilus influenzae* D15, the *Neisseria meningitidis* Omp85, and the *Pasteurella multocida* Omp87. These proteins are all believed to share a similar cellular function, and are believed to possess a similar structure. Homologues of this protein are believed to exist in all gram negative organisms (Voulhoux *et al*, 2003).

The bacterial surface antigen domain of the Omp85 family is a protein domain which is defined by an N-terminal sequence with a set of characteristic **Po**lypeptide **Tr**anslocation **A**ssociated (POTRA) motifs, and a C-terminal barrel domain consisting of 16 predicted β-strands (Sanchez-Pulido *et al*, 2003). Therefore, this family of proteins cannot be defined by a single consensus sequence or protein motif, and is hence more difficult to detect using the typical protein motif identifiers.

The protein was found to be encoded for in the *L. pneumophila* genome through the use of the protein families database (PFam) within the European Bioinformatics Institute (EBI) database. Database screening revealed a bacterial surface antigen domain which is present in outer membrane proteins such as the D15 protein of *H. influenzae*. Screening of the *L. pneumophila* genome with the protein sequence of the D15 antigen of *H. influenzae* revealed a 29% identity match with a gene, which had not yet been annotated in *L. pneumophila*. The alignment also showed that the gene was present in the *L. pneumophila* Philadelphia 1 genome between nucleotide positions 547,578-549,939 (2361bp in length). The gene encodes a novel *L. pneumophila* protein with an estimated molecular weight of 87 kDa, and was subsequently designated the name Omp87.

Further characterisation of this novel *L. pneumophila* protein will be required, and may provide further insight into our knowledge of *L. pneumophila*, including the mechanisms of its pathogenesis.

Further characterisation of the protein, including distribution studies and mutagenesis, will be performed in chapters 4 and 5 of this thesis.

CHAPTER IV

Legionella pneumophila

Outer Membrane Protein 87 (Omp87)

Characterisation and Analysis

4. Introduction

Homologues of the Omp87 protein are believed to exist in all gram-negative organisms (Voulhoux *et al*, 2003). Some of these homologues have been previously studied and characterised. These include the *Neisseria meningitidis* Omp85 (Genevrois *et al*, 2003), the *Haemophilus influenzae* D15 (Loosmoore *et al*, 1997) the *Shigella flexneri* Oma87 (Robb *et al*, 2001), and the *Pasteurella multocida* Oma87 (Ruffolo and Adler, 1996). There appears to be a consensus that the protein is involved in either lipid or protein transport to the outer membrane of the cell. In *Neisseria meningitidis*, Genevrois *et al* (2003) recently found that deletion of the *omp85* gene resulted in the depletion of lipopolysaccharides and phospholipids from the outer membrane of the bacterial cell, and a corresponding increase and accumulation of these lipids in the inner membrane of the cell. However Voulhoux *et al* (2003) believe that the observed decrease in LPS from the outer membrane of Omp85 mutants is more likely due to a defect in LPS transport machinery, and that Omp85 is instead involved in the insertion of proteins into the bacterial outer membrane. They found that depletion of the Meningococcal Omp85 resulted in a decrease in assembled proteins in the outer membrane, and a subsequent increase in unassembled forms of outer membrane proteins in the inner membrane of the cell.

Several groups have also analysed the potential of this relatively novel protein to serve as a vaccine candidate by studying its antigenicity and immunogenic properties.

Loosmore *et al* (1997) performed passive immunisation studies and showed that the D15 outer membrane protein of *Haemophilus influenzae* was able to protect infant rats against the development of bacteraemia due to *H. influenzae*. This D15 outer membrane protein was found to share an amino acid similarity of 75% with the Oma87 protein of *Pasteurella multocida* which may have been a factor which led Mitchison and co-workers to recently analyse the Oma87 protein of *Pasteurella multocida*, and its potential as a vaccine candidate (Mitchison *et al*, 2000). The group cloned fragments of the protein and analysed the ability of these fragments to protect chickens against a challenge from virulent *P. multocida*. Unfortunately, none of the fragments analysed were able to protect the immunised chickens against a challenge with a virulent strain of *P. multocida*.

4.1 Outline of this chapter

This chapter describes the characterisation and analysis performed of the Omp87 protein of *Legionella pneumophila* serogroup 1, strain AA100.

The analysis includes the initial PCR amplification of the *omp87* gene, through the development of novel PCR primers. It then continues with the DNA sequence determination of the *omp87* gene, followed by distribution studies of the *omp87* gene, by using Southern blotting in order to determine whether the *omp87* gene was also present in other species of *Legionella*, using a specific and novel *omp87* gene DNA probe.

Finally, the *omp87* gene was cloned into the vector pBluescript SKII-, in preparation for further analysis, such as mutagenesis studies (Chapter V).

4.2 Material and Methods

4.2.1 PCR amplification of the *omp87* gene of *L. pneumophila* AA100

The PCR was used to confirm the presence of the *omp87* gene.

4.2.1.1 Primer design

The primers designed for the amplification of the *omp87* gene were designated omp87a and omp87b. These were designed based on the published sequence of *Legionella pneumophila subsp. pneumophila* strain Philadelphia 1, available through the NCBI website, at http://www.ncbi.nlm.nih.gov/ with the sequence reference number of NC002942. The primer sequences are given in **Table 4.4.** The primers were designed with *Xba*I restriction sites at the 5′ end to facilitate cloning. The forward primer was designed approximately 300 bp upstream of the predicted start of the *omp87* gene to ensure that the promoter region of the protein was included in the amplification reaction.

4.2.1.2 PCR amplification

The *L. pneumophila* serogroup 1 strain AA100 used for the amplification reaction was kindly obtained from Prof. Yousef Abu Kwaik, at the Department of Microbiology and Immunolgy, University of Kentucky, Lexington, Kentucky, U.S.A.
The PCR amplification of the *omp87* gene was performed using the AmipliTaq DNA polymerase system (Roche Molecular Biochemicals). The optimised PCR conditions and mastermix components are given in **Table 4.1a and b.**

Table 4.1a - The optimised PCR conditions for amplification of *L. pneumophila omp87*

PCR Stage	Time (s) / Temp (°C)
STAGE 1	
Initial denaturation of DNA template	1 min / 94°C
STAGE 2	
Denaturation of DNA	30s / 94°C
Annealing of primers	30s / 62°C
Elongation	3min / 72°C
Number of cycles	35
STAGE 3	
Final elongation	5min / 72°C

Table 4.1b - The optimised PCR Master mix components

Reagent	**Volume**
Milli-Q water	33.5 µl
10 x PCR buffer	5 µl
$MgCl_2$	5 µl
dNTP's	1 µl
Primer 1	2 µl
Primer 2	2 µl
Taq polymerase	0.5 µl
DNA template	1 µl
Total Reaction Volume	**50 µl**

4.2.2 DNA Sequence determination of *L. pneumophila* AA100 *omp87*

Following the successful PCR amplification of the *L. pneumophila omp87* gene, the amplified product was prepared for DNA sequence determination. Due to the presence of slight non-specific background amplification, it was deemed necessary to purify the amplified product prior to sequencing. This was carried out by excising the DNA band from an agarose gel, using the QIAquick gel extraction kit (Qiagen, UK), according to the manufacturer's instructions.

Several amplified DNA samples were combined during the extraction process, in order to increase the amount of DNA. The sequencing reaction mixture, and sequencing reaction cycle conditions can be seen in **Table 4.2a and b**. Due to the length of the *omp87* gene fragment (2.7 kb) a second set of primers were designed for internal sequencing of the gene. The primers used for the sequencing reactions are described in **Table 4.3.**

The sequencing reactions were performed using an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Australia) in a Perkin-Elmer 2400 GeneAmp PCR system. Following the sequencing reaction, the sequencing products were precipitated using ethanol and sodium acetate, according to the manufacturer's instructions.

The DNA sequence determination was carried out by the DNA Sequencing Facility at Monash University, (Clayton campus), Victoria, Australia, using the ABI Prism 373 DNA Sequencer (Perkin-Elmer, Australia).

Table 4.2a - Sequencing reaction mixture used for DNA sequence determination of the *L. pneumophila omp87* gene

Reagent	Concentration	Volume
Ready reaction Premix	2.5 x	1 µl
Big Dye Sequencing Buffer	5 x	3.5 µl
Primer	-	20 ng (~1 µl)
DNA template (PCR product)	-	7.5 µl (~60ng)
MilliQ H_2O	-	7 µl
Final volume	**1 x**	**20 µl**

Table 4.2b - Sequencing reaction cycle conditions

	Time / Temp
STAGE 1	
Initial denaturation of DNA template	1 min / 96°C
STAGE 2	
Denaturation of DNA	10s / 96°C
Annealing of primers	5s / 50°C
Elongation	4min / 60°C
Number of cycles	25
STAGE 3	
Holding temperature (until purification)	4°C

4.2.2.1 Sequence analysis software and websites

Sequence analysis, comparisons and primer designing were performed using Clone Manager 6 software, version 6.00 (Sci Ed Central Software Inc.).

DNA sequence alignments and amino acid sequence searches were performed using both the **N**ational **C**entre for **B**iotechnology **I**nformation (NCBI) website, available at http://www.ncbi.nlm.nih.gov/ and the **Ex**pert **P**rotein **A**nalysis **Sy**stem (ExPASy) Molecular Biology Proteomics Server with Swiss-Prot/TrEMBL database, available at http://kr.expasy.org.

Table 4.3 Primers used in this study

Primer	Description	Source/Reference
omp87a	5′ TTTATCTAGAATCCCTATGCTGGATGGCGG 3′	This study
omp87b	5′ AATTTCTAGAGGTTGCGATATGTACAGCAC 3′	This study
omp87c	5′ TTAAAGAAATGGGGCTGGTC 3′	This study
omp87d	5′ TCCTGAAAATGATTACCGTT 3′	This study
Universal M13	5′ CACGACGTTGTAAAACGACGGCCAG 3′	Stratagene, U.S.A
Reverse M13	5′ CACCAGGAAACAGCTATGACCATG 3′	Stratagene, U.S.A

* Underlined sequence = *Xba*I restriction site.

4.2.3 Distribution studies of *omp87*: PCR and Southern blotting of the *L. pneumophila* AA100 *omp87* gene

In order to determine whether the *omp87* gene was universally present in all *Legionella* species and serogroups, distribution studies with the gene were performed. This was firstly carried out by PCR amplification of the gene in different species and serogroups. Southern blotting was then carried out on remaining non-reactive strains as a confirmatory test for the presence or likely absence of the gene.

4.2.3.1 *Legionella* species and serogroups included in this study

The *Legionella* serogroups and species included in this study were all kindly donated by Prof. Yousef Abu Kwaik, from the Department of Microbiology and Immunolgy, at the University of Kentucky, Lexington, Kentucky, U.S.A. The complete list of strains included can be seen in **Table 4.4**.

4.2.3.2 PCR amplification of the *omp87* gene of all *Legionella* species

The primers used for the amplification of the *omp87* gene in all other *Legionella* species and serogroups were the same as those used for the amplification of the internal fragment of the *omp87* gene for DNA sequence determination. These primers, designated omp87c and omp87d amplify a 1.2 kb region in the centre of the *omp87* gene. Their sequences are shown in **Table 4.3**.

The PCR conditions used for the amplification reaction of all *Legionella* strains were identical to those used for the amplification of the *L. pneumophila omp87* gene, and can be seen in **Table 4.1a and b**.

4.2.3.3 Southern blotting of the *omp87* gene

Genomic DNA from *Legionella* strains was extracted, following 48hr growth on BCYE agar, by the CTAB method (Ausbel *et al*, 1995). The concentrations of DNA obtained were determined by spectrophotometric absorbance at 260 nm. Ten µg of DNA was then digested overnight at 37°C, with the restriction enzyme *Hin*dIII, in a total volume of 200µl. Following the overnight digestion, the digested genomic DNA was precipitated using ethanol and 3M sodium acetate. The digested fragments were then subjected to agarose gel electrophoresis, on a 1.2% agarose gel, at a constant voltage of 70V for 2 hours.

The DNA in the gels was then transferred to nylon membranes overnight by capillary action, followed by cross-linking onto the membrane by 5 minutes of UV exposure in a transilluminator. The Southern blotting was performed under moderate stringency conditions.

4.2.3.4 Southern blotting DNA probe: design and labeling with DIG

The DNA probe used for detection of the *omp87* gene was designed based on the internal primers utilised for sequencing of the *omp87* gene of *L. pneumophila* serogroup 1. These primers were the same as those used for the PCR amplification of all *Legionella* species, discussed above in section 4.2.3.2. The amplified fragment obtained using these primers was considered appropriate for use as an *omp87* gene probe for detection of the gene in other *Legionella* species.

The fragment was therefore amplified from *L. pneumophila* serogroup 1 AA100, using the omp87c and omp87d primers. The optimised PCR conditions used were the same as those described in 4.2.1.2 **(Table 4.1a and b)**.

Several samples of amplified product were combined in order to increase the amount of probe DNA.

Labelling of the DNA probe was performed using the DIG DNA Labelling and Detection kit (Roche Molecular Biochemicals). All procedures were carried out according to the manufacturer's instructions using digoxigenin (DIG) –dUTP as the

label. The labelled DNA probe was denatured prior to being added to the nylon membranes in the blotting procedure, by boiling for 5 minutes.

Table 4.4 *Legionella* serogroups and species included in this study*

L. pneumophila serogroup 1 AA100	*Legionella longbeachae*
L. pneumophila serogroup 2	*Legionella spiritensis*
L. pneumophila serogroup 3	*Legionella dumoffii*
L. pneumophila serogroup 4	*Legionella gratiana*
L. pneumophila serogroup 5	*Legionella parisensis*
L. pneumophila serogroup 6	*Legionella santicrucis*
L. pneumophila serogroup 7	*Legionella cherrii*
L. pneumophila serogroup 8	*Legionella maceachernii*
L. pneumophila serogroup 9	*Legionella micdadei rivera*
L. pneumophila serogroup 10	*Legionella micdadei tatlock*
L. pneumophila serogroup 11	*Legionella moravica*
L. pneumophila serogroup 12	*Legionella wadsworthii*
L. pneumophila serogroup 13	

* All strains were obtained from Prof. Yousef Abu Kwaik from the Department of Microbiology and Immunolgy, at the University of Kentucky, Lexington, Kentucky, U.S.A.

4.2.4 Cloning of the *L. pneumophila* AA100 *omp87* gene

Following the successful PCR amplification of the *omp87* gene of *L. pneumophila*, it was cloned into the vector pBluescript SKII-. Following the PCR reaction, the amplified product was purified using the Promega PCR purification kit (Promega, U.S.A.) to remove salts and other impurities that may hinder the activity of enzymes. As the omp87a and omp87b primers were designed to include an *Xba*I restriction site, the PCR product could then be digested with *Xba*I. This was done in a total reaction volume of 20 µl, at 37°C for 1 hour. Following digestion, the *Xba*I enzyme in the reaction mixture was inactivated by phenol-chloroform treatment.
The pBluescript SKII- plasmid was digested under the same conditions, also with *Xba*I. In order to prevent self re-ligation, the vector was treated with calf intestinal phosphatase (CIP), at a concentration of 0.05 units, for 1 hour at 37°C.

4.2.4.1 DNA ligation

Ligations were performed in the ratio of one vector molecule to 2 insert molecules, and one vector molecule to 4 insert molecules. The ligation was performed with 10 U of T4 DNA Ligase and T4 DNA Ligase buffer (Boehringer Mannheim). The reaction mix was made up to 20 µl with sterile Milli-Q water and incubated at 16°C overnight. The mixture was then transformed into *E. coli* DH5α cells.

4.2.4.2 Electrotransformation

Electrocompetent *E. coli* DH5α cells were transformed with the ligation mixture by electrotransformation, using a Gene Pulser apparatus (Bio-Rad Gene Pulsar^{TM}), set at 25 µF and 1.25 kV, with the Pulse Controller set at 200 Ω. Immediately after pulsing 1 ml of SOC medium was added. The mixture was then transferred to a 1.5 ml polypropylene tube and incubated at 37°C for 1 hour. One hundred µl of this suspension was then plated out on LB agar plates, containing X-gal (20 mg/ml) and IPTG (20 mg/ml). The agar plates also contained ampicillin, at a concentration of 100 µg/ml.

4.2.4.3 PCR amplification of *omp87* clones

In order to screen white colonies for the desired plasmid construct, PCR amplification was carried out on the colonies. The white colonies were touched with a sterile toothpick from the agar plate and were inoculated directly into the reaction mixture, without any prior DNA extraction process. The PCR amplification reaction used was essentially identical to that used for the amplification of the *omp87* gene from *L. pneumophila* **(Table 4.1)** with the exception of the DNA template. In this case, the 1 µl of DNA was substituted for 1 µl of sterile milli-Q water. Primers used for the reaction were the pBluescript Universal M13 primers, which amplify the region of the multiple cloning site, and therefore the insert. The sequence of these primers is shown in **Table 4.3**.

4.2.4.4 Restriction digestion of *omp87* clones

Following the PCR amplification reaction, DNA from the colonies which displayed the expected insert sizes were digested with the restriction enzymes *Hin*dIII and *Xba*I. Three hundred ng of plasmid DNA was digested according to the conditions described in Chapter 2. The digestion was performed at 37°C for 2 hours.

4.3 Results

4.3.1 PCR amplification of the *omp87* gene in *L. pneumophila*

The *omp87* gene of *L. pneumophila* was amplified by PCR. The primers omp87a and omp87b amplified a fragment of ~ 2.7 kb, which included the 2.4 kb *omp87* gene, and 300 bp of DNA upstream of the gene. Optimisation of the PCR conditions were required in order to obtain the amplified product, and this was achieved mainly by optimisation of the primer annealing temperature. A gradient PCR, consisting of a temperature range spanning from 54-61°C was used **(Figure 4.1)**. A lower temperature range of 48-51°C was also analysed, but this gradient resulted in non-specific binding (results not shown).

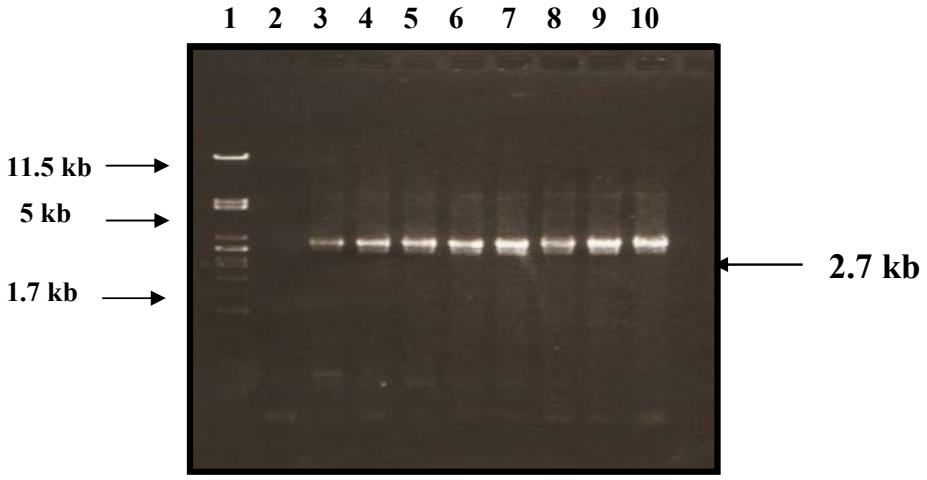


Figure 4.1. Gradient PCR amplification of the *L. pneumophila omp87* gene. Lane: 1, lambda x *Pst*I marker; Lane 2, negative control sample; Lanes 3-10, amplified product at annealing temperatures of 3: 54°C; 4: 55.4°C; 5: 56.3°C; 6: 57.4°C; 7: 58.4°C; 8: 59.5°C; 9: 61.4°C; 10: 62.1°C.

4.3.2 DNA Sequence determination of *L. pneumophila omp87*

The sequencing performed on the *L. pneumophila* AA100 *omp87* gene showed the gene to be ~ 2.4 kb in size. The sequence is shown in **Figure 4.2**. The sequencing chromatograms can be seen in **Appendix 3 (a and b)**. Analysis of this DNA sequence was performed through the **N**ational **C**entre for **B**iotechnology **I**nformation (NCBI) website, (http://www.ncbi.nlm.nih.gov/). A DNA sequence alignment, performed against the published *Legionella pneumophila* Philadelphia 1 strain, showed a similarity match of 98%. This sequence alignment is shown in **Figure 4.3**.

The analysis revealed that the DNA sequence encoded a protein of 786 amino acids, with a predicted molecular weight of 87 kDa. The protein sequence of Omp87 can be seen in **Figure 4.4**. A comparison of the *L. pneumophila* AA100 and Philadelphia 1 Omp87 DNA and protein sequences revealed that although there were 29 DNA base pair mismatches between the two sequences, these resulted in only 6 amino acid differences. This indicated that the Omp87 genes are highly conserved between these two strains.

Both the DNA and amino acid sequence of the Omp87 of *L. pneumophila* AA100 were submitted to GenBank (Accession number: DQ657353) (www.ncbi.nlm.nih.gov/GenBank).

A more in depth analysis of this amino acid sequence, using the **Ex**pert **P**rotein **A**nalysis **Sy**stem (ExPASy), at http://kr.expasy.org revealed that the protein contained a secretory signal sequence, of around 44 amino acids in length, situated at the N-terminal region of the protein. It was found to have an estimated pI value of 9.66. In addition, it was also predicted to contain a cleavage site positioned between amino acids 25-42. This cleavage site determines where the protein is cleaved following translocation within the cell. These features are outlined in **Figure 4.4.** An amino acid comparison was performed against a protein sequence database, also through the NCBI website. This BLAST analysis revealed a large number of matches with previously identified proteins. **Table 4.5** summarises some of these matches, and these have been ranked in order of their identity % matches.

The E value, or 'expected' value of the match is also included. This E value is a parameter which represents the number of times the particular match would be expected to occur purely by chance. Therefore, the lower the E-value, and the closer it is to zero, the more likely the match is significant, and not purely a chance event.

In the case where two matches have the same % identity match, they were then ranked according to their E value, with the smaller E value ranked higher.

TTTATCTAGAATCCCTATGCTGGATGGCGGTCATCTTTTGTATTATGTCTTGGAAATCAT
AAGAAGAAAGCCATTATCGGACGGAGTTAAATCTGTCGCGTTCTATTTTGGATTATTACT
GTTGGTTGCCTTAATGTTTGTTGCTCTTAGTAATGATATATCAAGATTAACCAGTTAGGA
TTTGGCGAGAGTAATTCTAATTTCTGTATGATTAATGAATGAAATGATGTTTTAGCGACA
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ATGGTTTTTAATTTCATAAGTGCGCGTAGTGCTGGACGTAAAATAATAATGAAAAAAGTC
AGTAATAAATTAATATTAGGTGTTTGTTGTTCTACTCTTTTAGCTTGGTCATCCCAAACC
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ACAGGTACGGTATTAAACTATATGCCTGTGCAAGTGGGTGAGGAAATCAGTTCCAGCTCA
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TTTCCCGCGATTAATGCTGAACCTAGAATAGATGAAAATAATAAAACAGTATTTATTACT
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TTTAATGCATCCGTTAACCAACGCAATTTTATGGGAAGTGGACGTTCCATGGGAGCTGCT
TTCAATGCAAGTCAATGGGGGCAAGACTACTCTTTTAACTATTATAATCCGTTCTATACC
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GGTGAGAAAAGCAGCTTTCAATTGGGATATGGTTATCAAGATATCAATATTAAGTCAGTG
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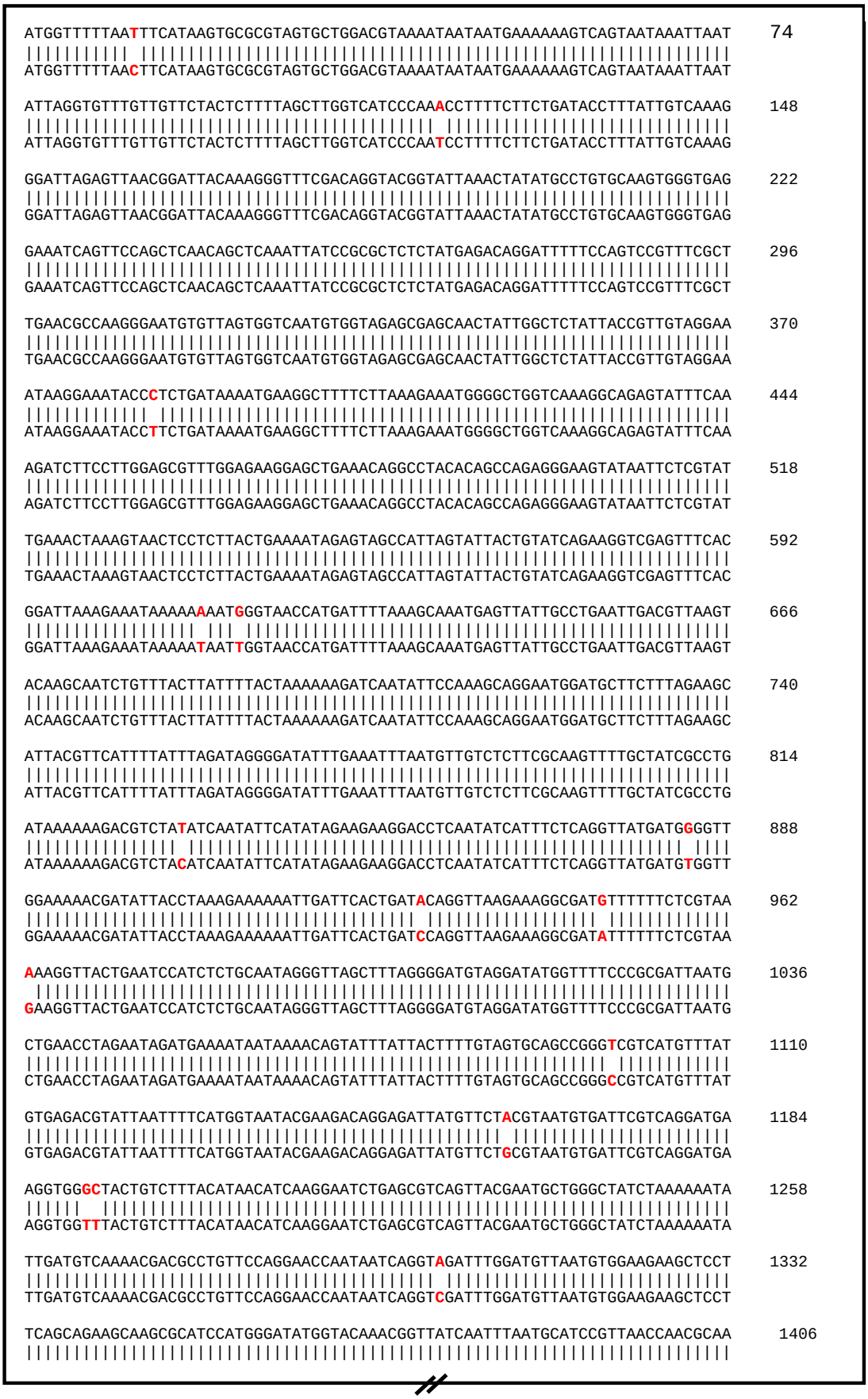
CCGATGCGTTATTCAGCTGGTGTGTCATTAGAATGGCGCTCACCTTTTGGTCCATTGTCT
TTTAGCTTGGCTAAGGCATTGAATCCACAGCCATTGGATCAGACTCAGCTCTTCCAATTT
GCTCTTTCCTCAGGTTTT**TAG**AGGTATTATGGATTAGAGTAAGCAGATTTTAGTATTTTA
AAAACTAGTGCTGTACATATCGCAACCTCTAGAAATT

Figure 4.2. DNA sequence of the *L. pneumophila omp87* gene. The highlighted base pairs indicate the start of the gene **(ATG)** and the end of the gene **(TAG)**.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GGATTAGAGTTAACGGATTACAAAGGGTTTCGACAGGTACGGTATTAAACTATATGCCTGTGCAAGTGGGTGAG 222
GGATTAGAGTTAACGGATTACAAAGGGTTTCGACAGGTACGGTATTAAACTATATGCCTGTGCAAGTGGGTGAG
GAAATCAGTTCCAGCTCAACAGCTCAAATTATCCGCGCTCTCTATGAGACAGGATTTTTCCAGTCCGTTTCGCT 296
GAAATCAGTTCCAGCTCAACAGCTCAAATTATCCGCGCTCTCTATGAGACAGGATTTTTCCAGTCCGTTTCGCT
TGAACGCCAAGGGAATGTGTTAGTGGTCAATGTGGTAGAGCGAGCAACTATTGGCTCTATTACCGTTGTAGGAA 370
TGAACGCCAAGGGAATGTGTTAGTGGTCAATGTGGTAGAGCGAGCAACTATTGGCTCTATTACCGTTGTAGGAA
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ATAAGGAAATACCTTCTGATAAAATGAAGGCTTTTCTTAAAGAAATGGGGCTGGTCAAAGGCAGAGTATTTCAA
AGATCTTCCTTGGAGCGTTTGGAGAAGGAGCTGAAACAGGCCTACACAGCCAGAGGGAAGTATAATTCTCGTAT 518
AGATCTTCCTTGGAGCGTTTGGAGAAGGAGCTGAAACAGGCCTACACAGCCAGAGGGAAGTATAATTCTCGTAT
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GGATTAAAGAAATAAAAATAATTGGTAACCATGATTTTAAAGCAAATGAGTTATTGCCTGAATTGACGTTAAGT
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ATTACGTTCATTTTATTTAGATAGGGGATATTTGAAATTTAATGTTGTCTCTTCGCAAGTTTTGCTATCGCCTG 814
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CTGAACCTAGAATAGATGAAAATAATAAAACAGTATTTATTACTTTTGTAGTGCAGCCGGGTCGTCATGTTTAT 1110
CTGAACCTAGAATAGATGAAAATAATAAAACAGTATTTATTACTTTTGTAGTGCAGCCGGGCCGTCATGTTTAT
GTGAGACGTATTAATTTTCATGGTAATACGAAGACAGGAGATTATGTTCTACGTAATGTGATTCGTCAGGATGA 1184
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AGGTGGGCTACTGTCTTTACATAACATCAAGGAATCTGAGCGTCAGTTACGAATGCTGGGCTATCTAAAAAATA 1258
AGGTGGTTTACTGTCTTTACATAACATCAAGGAATCTGAGCGTCAGTTACGAATGCTGGGCTATCTAAAAAATA
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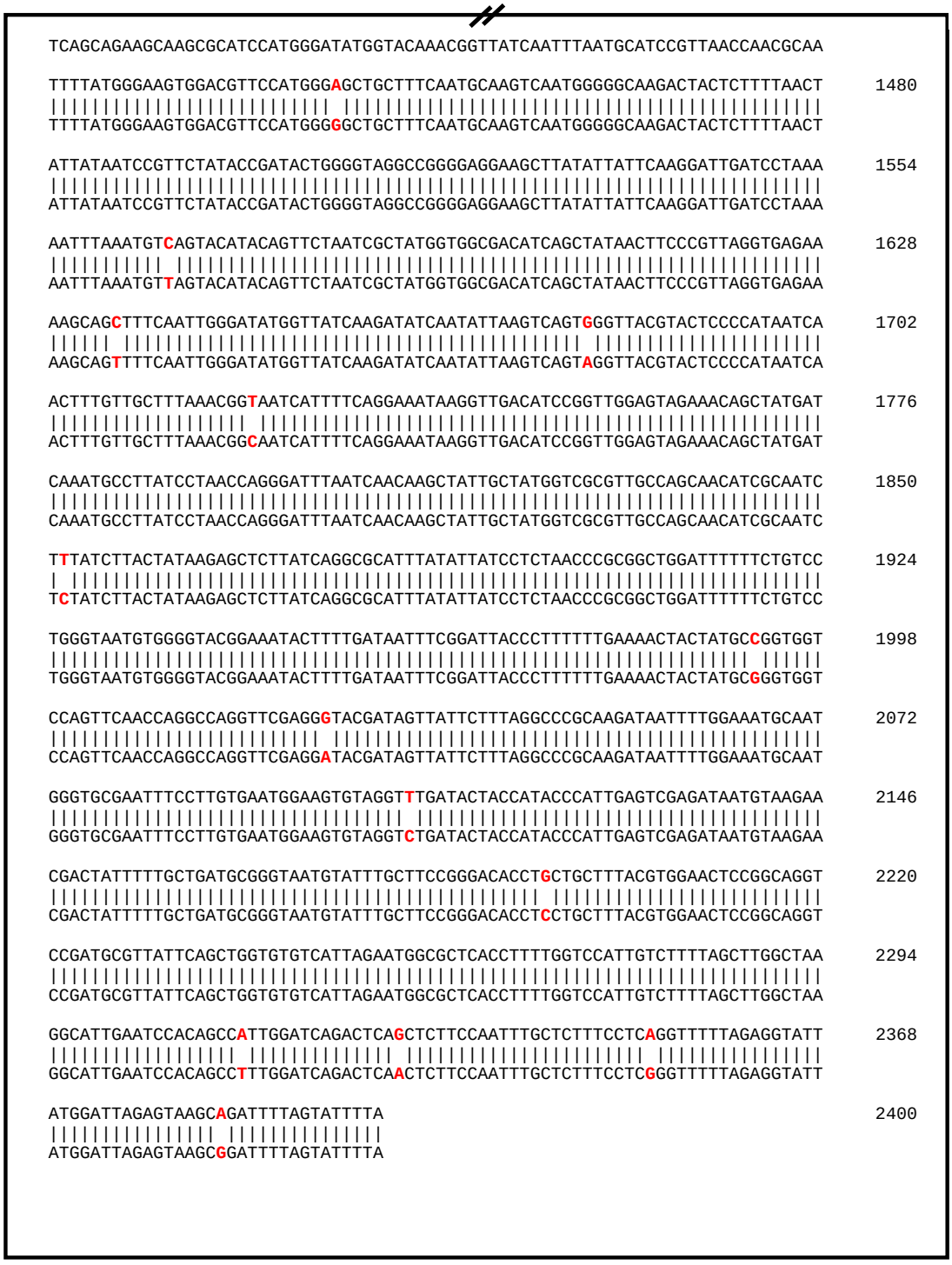


Figure 4.3. DNA sequence alignment of *L. pneumophila* AA100 *omp87* and the published *L. pneumophila* Philadelphia 1 strain (from NCBI database).

Base pair match= 2371/2400 (98%), Gaps = 0/2400 (0%)

Figure 4.4. Protein sequence of the *L. pneumophila* AA100 Omp87 protein. The protein consists of 786 amino acids. The pink shaded box represents the secretory signal sequence, which is situated at the N-terminal region and has an estimated length of 44 amino acids. The purple shaded box represents the predicted region of the cleavage site, which is situated between amino acids 25-42.

Table 4.5. Summary of amino acid BLAST analysis results. The table shows protein matches with similar amino acid sequences to *L. pneumophila* AA100 Omp87. The % identity match, and the E value of the matches are also included.

NCBI Reference no.	Bacterial species/strain	Protein Match	Identity (% Match)	E value
002942.5	*Legionella pneumophila subsp. pneumophila* str. Philadelphia 1,	Outer membrane protein	770/786 (98%)	0.0
006369.1	*Legionella pneumophila* str. Lens	Hypothetical protein	770/786 (97%)	0.0
007484.1	*Nitrosococcus oceani* ATCC 19707	Outer membrane protein	328/778 (42%)	$5e^{-162}$
007614.1	*Nitrosospira multiformis* ATCC 25196	Surface antigen (D15)	302/756 (39%)	$3e^{-142}$
007404.1	*Thiobacillus denitrificans* ATCC 25259	Surface antigen (D15)	297/747 (39%)	$2e^{-141}$
007947.1	*Methylobacillus flagellatus* KT	Surface antigen (D15)	296/784 (37%)	$3e^{-143}$
004757.1	*Nitrosomonas europaea* ATCC 19718	Bacterial surface antigen (D15)	280/756 (37%)	$2e^{-134}$
AABQ070-00001.1	*Pseudomonas aeruginosa* C3719	Outer membrane protein/protective antigen OMA87	291/780 (37%)	$3e^{-130}$

NCBI Reference no.	Bacterial species/strain	Protein Match	Identity (% Match)	E value
N007005.1	*Pseudomonas syringae pv. syringae* B728a	Surface antigen (D15):Surface antigen variable number	291/804 (36%)	$5e^{-132}$
AAAL020-00004.1	*Xylella fastidiosa* Dixon ctg90	Surface antigen (D15):Surface antigen variable number	278/771 (36%)	$2e^{-129}$
005773.3	*Pseudomonas syringae pv. phaseolicola* 1448A	Outer membrane protein, OMP85 family	286/798 (35%)	$3e^{-130}$
004129.6|	*Pseudomonas fluorescens* Pf-5	Outer membrane protein, OMP85 family	289/809 (35%)	$2e^{-130}$
004459.1	*Vibrio vulnificus* CMCP6	Outer membrane surface antigen	278/807 (34%)	$9e^{-118}$
AAKG01-000001.1	*Vibrio cholerae* O395	Outer membrane protein/protective antigen OMA87	271/813 (33%)	$3e^{-114}$
AALB010-00001.1	*Shewanella putrefaciens* CN-32 ctg164	surface antigen (D15):Surface antigen	268/832 (32%)	$2e^{-113}$

4.3.3 Distribution studies of the *omp87* gene

4.3.3.1 PCR amplification of the *omp87* gene in all *Legionella* strains

PCR amplification of the *omp87* gene was successful in a number of *Legionella* strains. The gene was amplified in all *Legionella* serogroups 1-13, except serogroups 2, 3, 4, 5 and 8. Results of the amplification of the majority of strains can be seen in **Figure 4.5,** displaying the expected 1.2 kb amplified product. All PCR reactions were repeated multiple times. This resulted in an amplification product for serogroup 2, whilst serogroups 3, 4, 5 and 8 remained negative.

Overall therefore, the expected amplification of the *omp87* gene was shown in *L. pneumophila* serogroups 1, 2, 6, 7, 9, 10, 11, 12 and 13.

The *omp87* gene of other *Legionella* species was then amplified by PCR. These included *L. longbeachae, L. spiritensis, L. dumoffii, L. gratiana, L. parisensis, L. santicrucis, L. cherrii, L. maceachernii, L. moravica, L. micdadei rivera, L. micdadei tatlock* and *L. wadsworthii.* Results of the PCR amplification can be seen in **Figure 4.6.** The PCR experiment was repeated 3 times. Amplification products were only observed in the species *L. longbeachae* and *L. gratiana*.

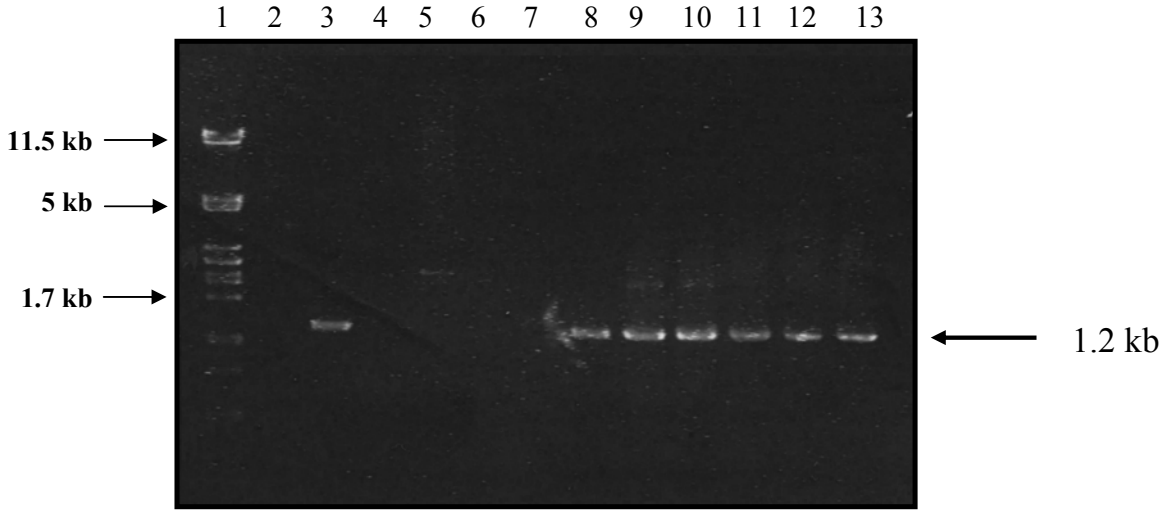


Figure 4.5. PCR amplification of the *omp87* gene in *Legionella pneumophila* serogroups 1-13. Lane: 1, lambda x *Pst*I marker; Lane 2, negative control; Lane 3, LP1; Lane 4, LP2; Lane 5, LP3; Lane 6, LP4; Lane 7, LP5; Lane 8, LP6; Lane 9; LP9; Lane 10, LP10; Lane 11, LP11; Lane12, LP12; Lane 13, LP13. (LP serogroups 7 and 8 were not included in this amplification reaction).

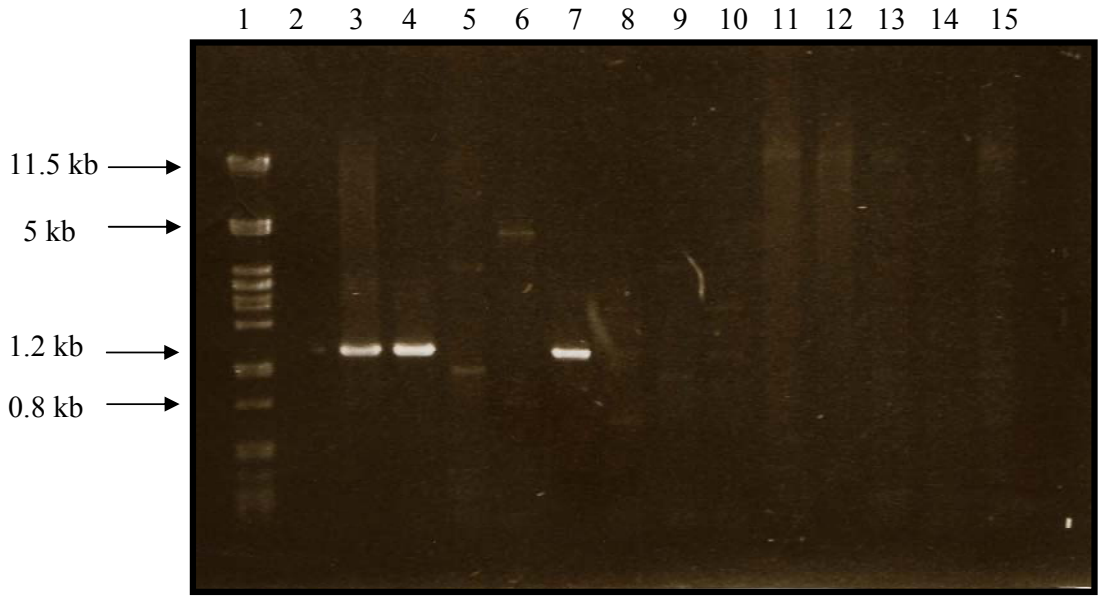


Figure 4.6 PCR amplification of the *omp87* gene in other *Legionella* species. Again, the 1.2 kb amplified product is evident. Lane 1, lambda x *Pst*I marker*;* Lane 2*,* negative control; Lane 3, positive control *L. pneumophila 1*; Lanes 4-15, *L. longbeachae, L. spiritensis, L. dumoffii, L. gratiana, L. parisensis, L. santicrucis, L. cherrii, L. maceachernii, L. moravica, L. micdadei rivera, L. micdadei tatlock* and *L. wadsworthii.*

4.3.3.2 Southern Blotting of the *L. pneumophila* AA100 *omp87* gene

4.3.3.2.1 DNA Probe: Design and labelling with DIG

The DNA probe used for the Southern blotting study was designed based on the same primer pair used for the amplification of part of the *omp87* gene in all *Legionella* species. A fragment of 1.2 kb was amplified from *L. pneumophila* AA100. Several samples were combined and purified so as to obtain a higher concentration of DNA for production of the probe. The PCR amplification of the *L. pneumophila omp87* gene for probe production can be seen in **Figure 4.7.**

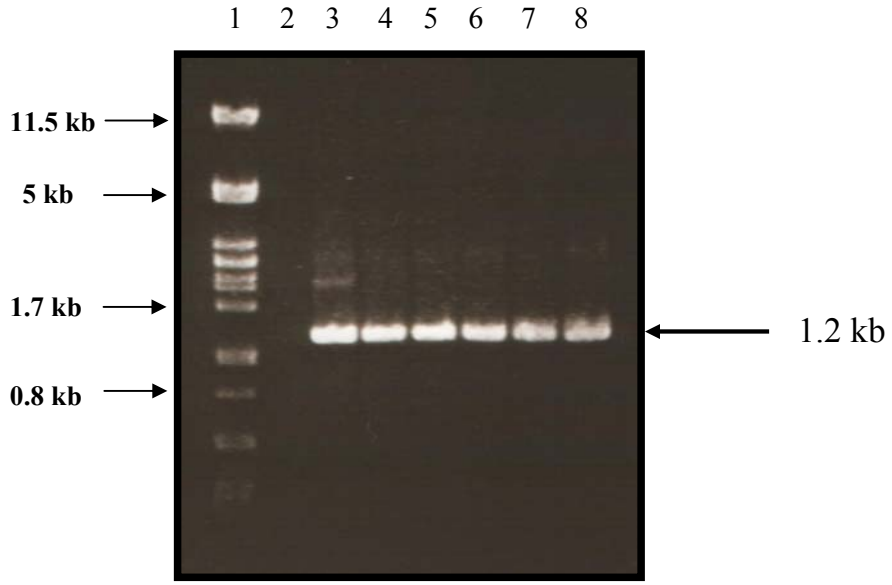


Figure 4.7. PCR amplification of the 1.2 kb fragment of the *L. pneumophila* AA100 *omp87* gene for the Southern blotting DNA probe. This band was excised from the gel, purified, and labelled with DIG using the DIG DNA Labelling and Detection kit (Roche Molecular Biochemicals).

4.3.3.2.2 Southern blotting

The Southern blotting reaction was performed under moderate stringency conditions, on all *L. pneumophila* serogroups 1-13, even though the majority of these had already reacted positively by PCR for the *omp87* gene. In addition, other *Legionella* species were included in this experiment. The results of the Southern blotting can be seen in **Figures 4.8 and 4.9**

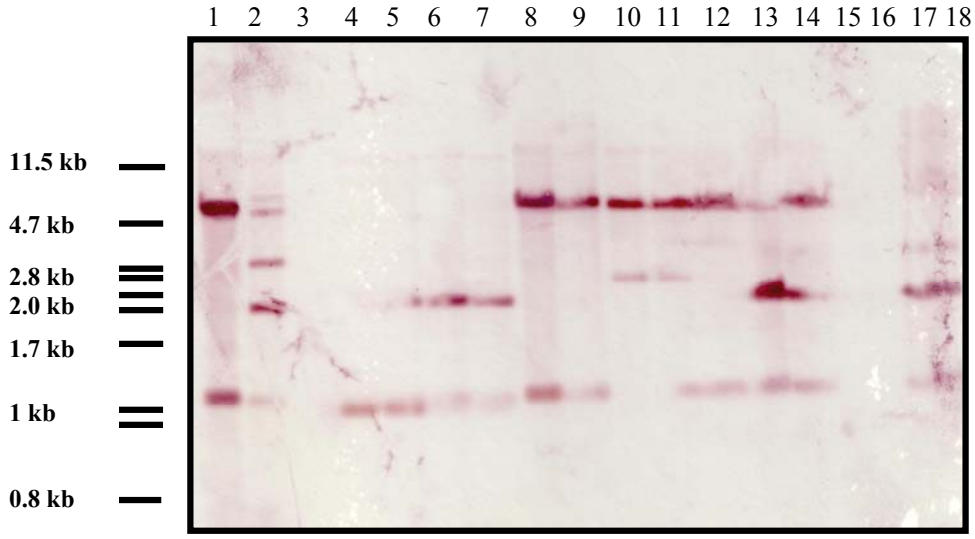


Figure 4.8. Southern blotting reaction of *L. pneumophila* serogroups 1-13 and other *Legionella* species. Lanes: 1-13: *L. pneumophila* serogroups 1-13 respectively; Lane 14: *L. spiritensis*; Lane 15: *L. dumoffii*; Lane 16: *L. gratiana*; Lane 17: *L. cherii*; Lane 18: *L. micdadei tatlock.*

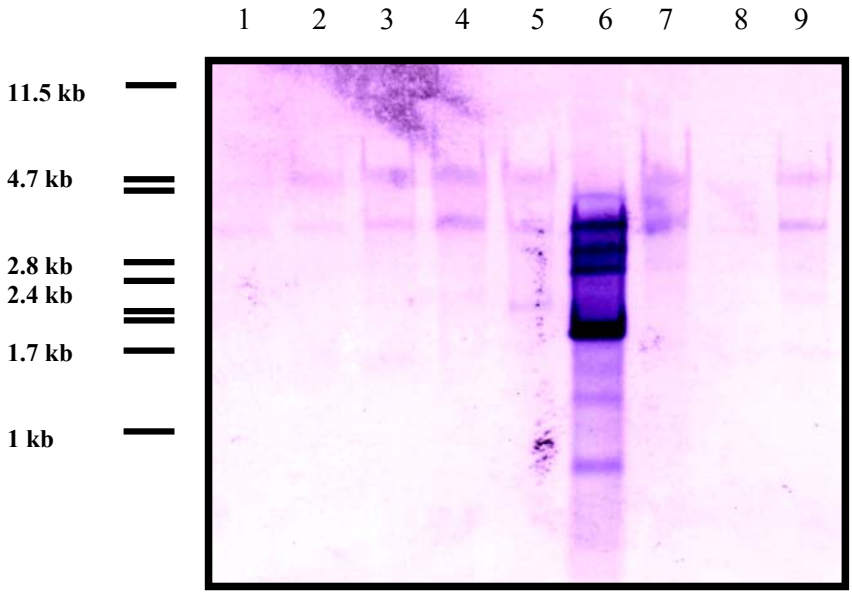


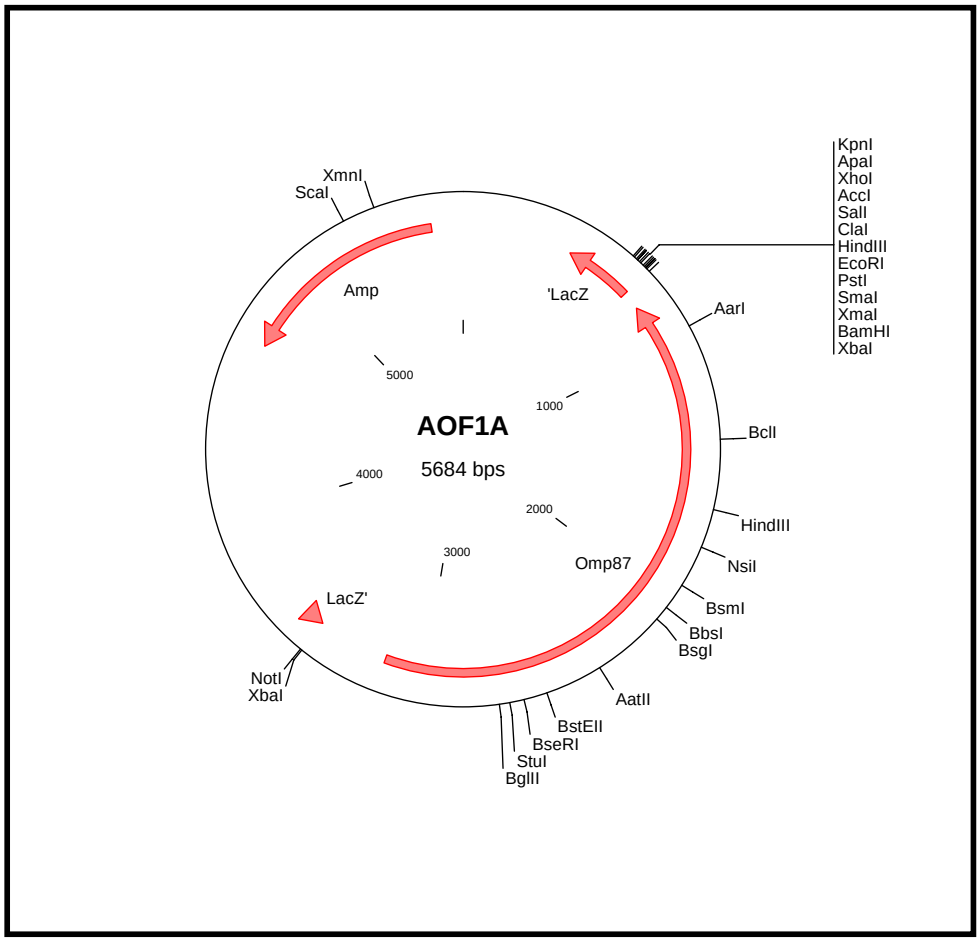
Figure 4.9 Southern blotting reaction of *Legionella* species. Lane 1: *L. wadsworthii*; Lane 2: *L. santicrucis*; Lane 3: *L. maceachernii*; Lane 4: *L. moravica*, Lane 5: *L. cherrii*; Lane 6: *L. parisensis*; Lane 7: *L. spiritensis*; Lane 8: *L. santicrucis*; Lane 9: *L. micdadei rivera.*

Figure 4.9 shows that all samples reacted with the probe, except *L. pneumophila* serogroup 3 (lane 3), *L. dumoffii*; (lane 15) and *L. gratiana* (lane 16). Figure 4.10 shows that all samples reacted with the probe, although the amount of reactivity varies between species.

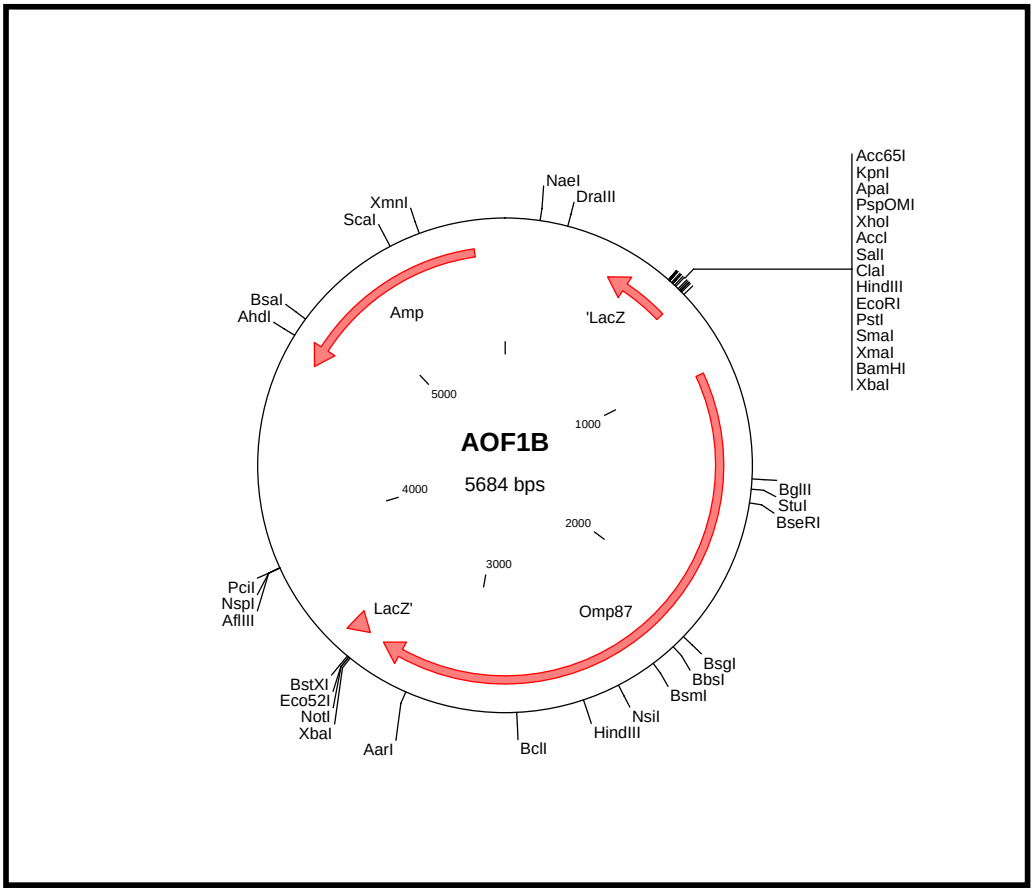
4.3.4 Cloning of the *L. pneumophila* AA100 *omp87* gene

Following the elecrotransformation and growing of colonies, the blue/white screening principle was used to select colonies which had obtained the *omp87* gene fragment. Seven white colonies (T1-T7) were firstly screened by PCR to determine which of these harboured the cloned fragment. The pBluescript Universal M13 primers were used for

the amplification reaction. These primers amplify the multiple cloning site (MCS) region of the plasmids. The vectors representative of these clones can be seen in **Figures 4.10a and b**. The results from the amplification reaction can be seen in **Figure 4.11.**



(a)



(b)

Figure 4.10. pBluescript vector harbouring the 2.7 kb amplified fragment containing the *L. pneumophila* AA100 *omp87* gene in a) the forward orientation and b) the reverse orientation. Genes are indicated by red arrows. Amp: Ampicillin gene; Omp87: Omp87 gene; LacZ: LacZ gene.

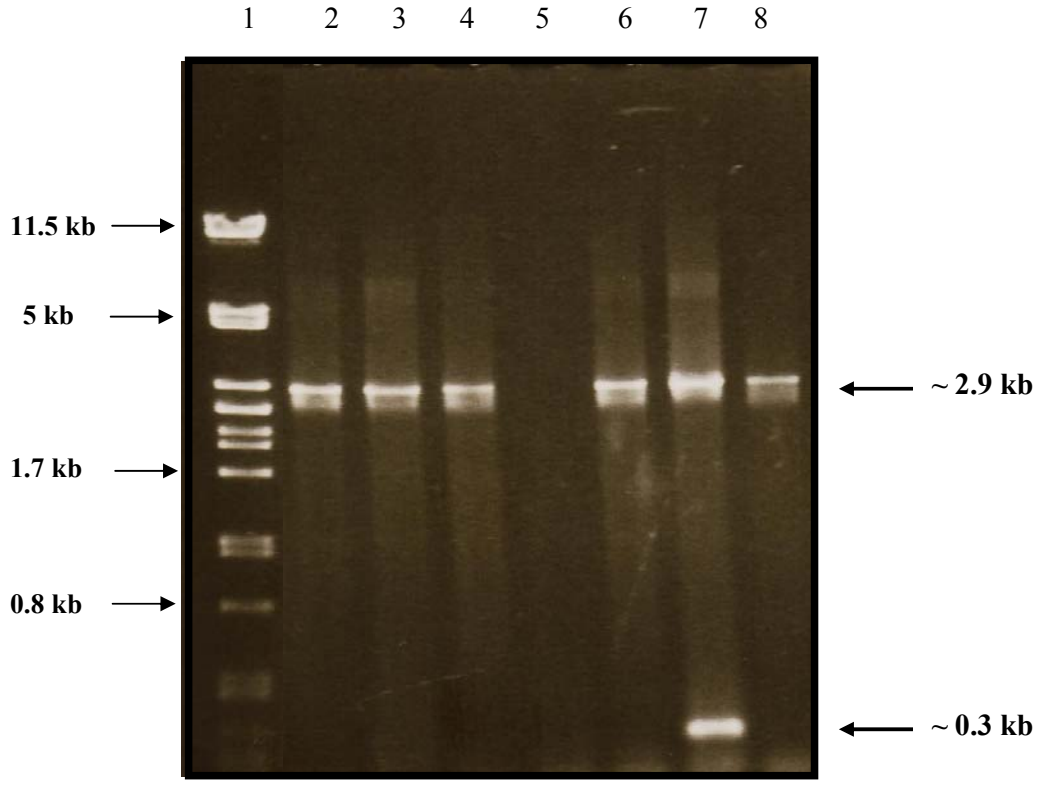


Figure 4.11 PCR Amplification reaction of the *omp87* gene clones. Lane 1: lambda x *Pst*I marker*;* Lanes 2-8: White colonies T1 - T7 selected for screening of *omp87* clones.

Following the PCR reaction, it was observed that 5 of the 7 samples amplified the expected product size of ~2.9 kb (T1, T2, T3, T5 and T7). This size includes the inserted *omp87* gene fragment (~2.7 kb) plus the ~ 200-300 bp amplified from the plasmid's multiple cloning site. The T6 clone (lane 7) appeared to contain the 2.9 kb insert, but also contained an additional band of around 300 bp. It was predicted that this sample may have included both a white and a blue colony, hence producing the observed

banding pattern. DNA from the 5 positive clones were then digested with restriction enzymes to determine the orientation of the insert. Cells were therefore re-grown, and plasmid minipreps were performed in order to isolate the pBluescript plasmid from the cells. Following this, plasmids were digested with the appropriate enzymes and digestion products were analysed by agarose gel electrophoresis. Results of the restriction digestion of each of the clones is shown in **Figure 4.13**.

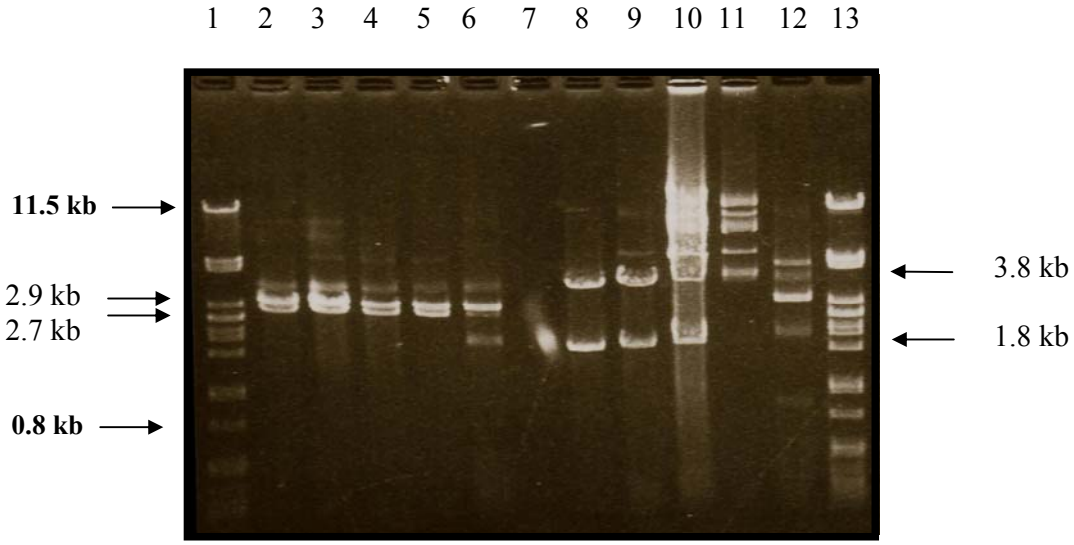


Figure 4.12. Restriction digest pattern of *omp87* clones. Lane 1: lambda x *Pst*I marker; Lanes 2-6: Plasmids isolated from transformants T1, T2, T3, T5 and T7 digested with *Xba*I; Lanes 8-12: Plasmids isolated from transformants T1, T2, T3, T5 and T7 digested with *Hin*dIII; Lane 13: lambda x *Pst*I marker.

DNA from clones digested with *Xba*I were expected to produce the following fragment sizes:

Forward orientation: 2.7 kb and 2.9 kb

Reverse orientation: 2.7 kb and 2.9 kb

DNA from clones digested with *Hin*dIII, however, were expected to produce the following fragment sizes:

Forward orientation: 4.7 kb and 0.9 kb

Reverse orientation: 3.8 kb and 1.8 kb

The *Hin*dIII enzyme therefore produces a differential banding pattern which enables for the determination of insert orientation. As seen from the gel in **Figure 4.12**, Lanes 2-6, digested with *Xba*I do not give an indication of insert orientation. Lanes 8-12 however, display a difference in banding size. Lanes 8-10 show the predicted banding pattern for inserts in the reverse orientation. Lanes 11 and 12 however, (samples 4 and 5) appeared partially undigested, and were therefore repeated.

The repeated restriction digest was performed using the enzymes *Bgl*II and *Xho*I. This was a double digestion, using both enzymes simultaneously. This reaction was again performed in a 20 µl reaction volume. The sizes of the inserts expected using this restriction enzyme combination are as follows:

Forward orientation: 2 kb and 3.6 kb.

Reverse orientation: 4.8 kb and 0.8 kb

Results of the restriction digestion are shown in **Figure 4.13.**

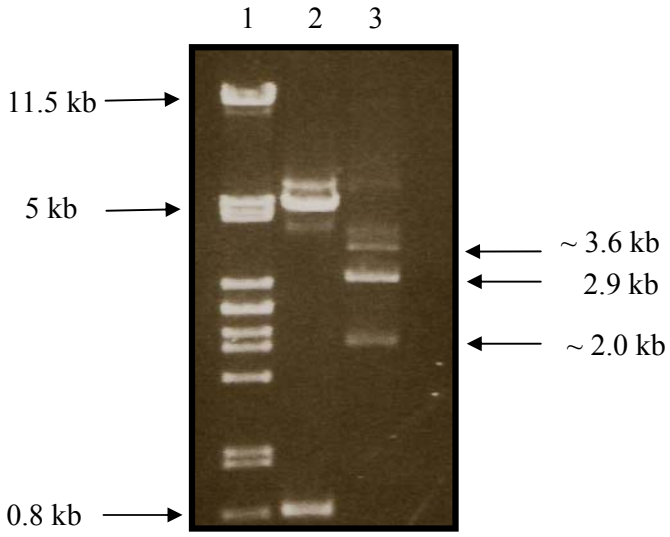


Figure 4.13. Restriction digest of partially digested clones 4 and 5. Lane 1: lambda x *Pst*I marker; Lane 2: *omp87* clone 4 x *Bgl*II and *Xho*I; Lane 3: *omp87* clone 5 x *Bgl*II and *Xho*I.

As can be seen from Figure 4.14, the plasmid in lane 2 is in the reverse orientation, as the digestion pattern revealed the expected product sizes of 4.8 kb and 0.8 kb. The clone in lane 3 revealed some bands of the sizes expected from a forward orientation insert, but also contained several other bands. Among these, was the 2.9 kb fragment indicative of an empty pBluescript vector. This indicated that the clone was indeed a mixture of both a forward orientation insert, and an empty pBluescript vector. In order to separate out the successful clone, the mixture was re-grown and again plated out on agar media. Due to the low success of clones with inserts in the forward orientation, it was thought that this may be due to the high stress exerted on the cells by the expression of the entire *omp87* gene. The cells were therefore grown without the addition of the inducer IPTG, so as to minimise the stress on the cells and increase the likelihood of recovering a plasmid with the *omp87* gene in the forward orientation. Following re-growth, colonies were again harvested, and the isolated plasmids were again digested with *Bgl*II and *Xho*I. Results of the digestion are shown in **Figure 4.14**.

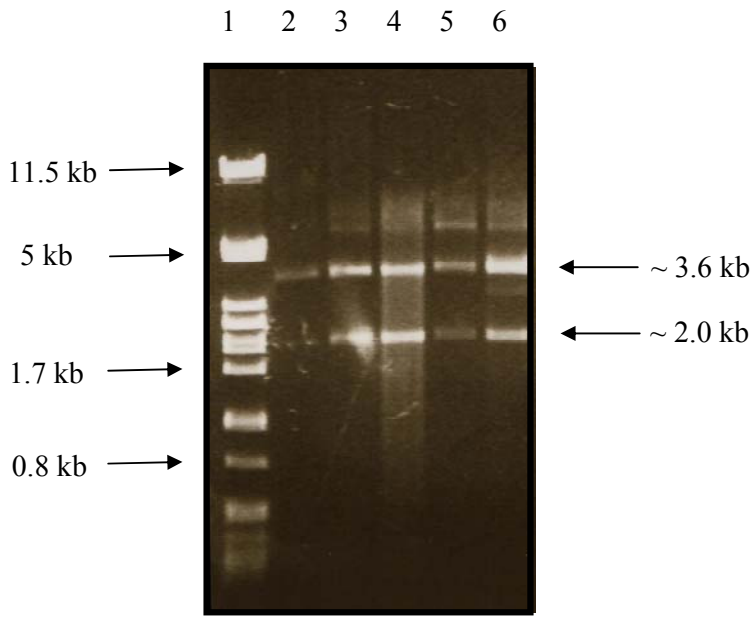


Figure 4.14. Restriction digestion of clones with *Bgl*II and *Xho*I. Lane 1: lambda x *Pst*I marker; Lanes 2-6: *omp87* clones with forward orientation inserts.

The digested clones were found to contain the predicted sizes of clones with inserts in the forward orientation. These clones were therefore used for further experiments.

4.4 Discussion

Sequence analysis performed on the *omp87* DNA sequence of *L. pneumophila* AA100, showed that this gene was 98% identical to the publicly available genome sequence of *Legionella pneumophila subsp. pneumophila* strain Philadelphia 1.
Comparison of the amino acid sequence of the *L. pneumophila* AA100 Omp87 with proteins in the Genbank database, showed that the protein was similar to outer membrane proteins, particularly belonging to the Omp85 family, of numerous other organisms. These included *Nitrosococcus oceani* (42% similarity), *Nitrosospira multiformis* (39% similarity) and *Thiobacillus denitrificans* (39% similarity).

The Omp87 protein was found to be 786 amino acids (87 kDa) in size, and was predicted to contain a secretory signal sequence of around 44 amino acids in length, situated at the N-terminal region of the protein. It was also predicted to contain a cleavage site, situated between amino acids 25-42, also at the N-terminal end. These findings were similar to those of Manning *et al* (1998), who characterised the Omp85 of *N. gonorrhoea* and *N. meningitidis.* They found that the Omp85 of these organisms was 792 amino acids (87 kDa) in size, and contained a secretory signal at the N-terminal end, with a cleavage site at around 60 amino acids from the protein N-terminal end. Similarly, characterisation of the D15 outer membrane protein, also belonging to the Omp85 family, of *H. influenzae* by Flack *et al* (1995), found that the protein was 797 amino acids (85kDa) in size and contained a secretory signal at the N-terminal end of around 19 amino acids, with a predicted cleavage site at the end of this region, between amino acids 19 and 20.

The distribution studies performed on the gene revealed that the *omp87* gene was present in most, but not all, serogroups and species of *Legionella*. From these results, however, we can not conclude that the gene is not present in some strains. Sequence variability will play a large role in the PCR and the Southern blotting reactions. Small variations at the locations of primer binding in the sequence will lead to false negative PCR reactions, and sequence variability, together with stringency conditions, can alter Southern blotting results. Also, the quality of the genomic DNA digestion proved to be another critical parameter. If DNA-ases are present in the DNA samples it will result in

degradation of the DNA leading to false negative results. Insufficient DNA blotted onto the nylon membrane can also lead to false negative reactions.

Several differences between the PCR and Southern blotting results were seen. Interestingly, the species *L. gratiana* was found to be positive by PCR, displaying the 1.2 kb fragment, but was one of the few samples which was not reactive by Southern blotting. This indicated that samples must be confirmed with numerous experiments before they can be explicitly labeled positive or negative, as many factors may interfere with, and hinder results.

The Southern blotting revealed that some *Legionella* species showed a similar banding pattern, however many showed different banding patterns that were unique. Some of the *L. pneumophila* serogroups 1-13 showed very similar patterns, which were characteristic of *L. pneumophila* serogroup 1, for which the DNA probe was designed. The *L. pneumophila* serogroups 1-13 are believed to be genetically closely related, hence would be expected to show a similar banding pattern.

Based partly on the *L. pneumophila* serogroup 1 AA100 strain used in this study, and the published *L. pneumophila subsp. pneumophila* strain Philadelphia 1 DNA sequence, the sizes of the expected fragments were calculated. However, the actual fragment sizes observed in the Southern blotting do not entirely correlate with the calculated ones. This is most likely due to the slight variations in the DNA sequence of the reference strain (*L. pneumophila subsp. pneumophila* strain Philadelphia 1) and the strain used in this experiment. This seems likely as the fragments obtained still match partially with the predicted *Hin*dIII restriction fragments. Small differences in DNA sequence are sufficient to alter the genomic DNA restriction digest pattern of the sample, which will alter the sizes of the fragments reacting with the probe (Lodish *et al*, 2004).

The main aims of the Southern blotting and PCR reactions were to analyse the distribution of the gene, and determine if its presence throughout the *Legionella* genus was widespread. The variations in banding patterns observed in the Southern blotting are therefore not as important as the fact that there is reactivity with almost all strains. These results therefore gave a good indication that the *omp87* gene indeed appears to be present in most *Legionella* serogroups and species. Further testing would be required on the negative strains in order to definitively determine the presence or absence of the

gene. Lowering the stringency conditions of the Southern blot would also probably result in more positive reactions.

Cloning of the *L. pneumophila* AA100 *omp87* gene proved to be quite laborious, and obtaining a clone with the insert in the forward orientation was difficult. Numerous clones were obtained with their insert in the reverse orientation, but only a mixed colony (one containing an empty vector and one containing the vector with insert) was found to have a forwardly inserted *omp87* gene.

The complete cloned *omp87* gene may have placed significant stress on the cell. When IPTG was added to plates during the blue/white screening process, this enhanced the expression of the *lacZ* promoter, and hence expression of the *omp87* gene insert. This may then have been too strenuous for the cell. The entire *omp87* gene was cloned so that it also contained the DNA encoding for the secretory signal sequence of the protein. This sequence therefore may lead to the protein's translocation to another site within the cell, following protein translation and processing. The Omp87 is an outer membrane protein, therefore meaning that its translocation would be to the outer membrane of the bacterial cell. An accumulation of too many proteins in the outer membrane of the cell would most likely have a detrimental effect on the cell, and would probably therefore have a negative impact on cell propagation and survival. Mitchison *et al*, (2000) also had difficulty cloning the entire *oma87* gene of *Pasteurella multocida*, and resorted to cloning shorter fragments of the gene, despite several attempts, and the use of several different cloning vectors.

Re-growing the mixed colony containing the *L. pneumophila omp87* gene on plates without IPTG may perhaps have been the critical element in obtaining the clone with the forward insert, as there would not have been any induction and over-expression of the gene, besides the normal or 'leaky' expression that is to be expected. The mixed growth would likely have reduced the amount of IPTG available to the clone carrying the construct with the *omp87* gene in the forward orientation.

By growing the colonies on plates without IPTG, the blue-white screening tool was therefore not available to aid with screening of colonies. This was not a problem, however, and simply meant that more colonies needed to be screened, and that several of these colonies were found to contain the empty pBluescript plasmid.

Another possibility may have been to clone part of the *omp87* gene, omitting the secretory signal sequence. In this way, the majority of the gene would be present, but the

lack of signal peptide sequence would prevent export of the protein to the outer membrane of the cell, thus reducing the stress placed on the cell, due to protein accumulation in the cells outer membrane. This approach may have been an option and would have been undertaken if this cloning attempt had not been successful.

The *omp87* construct produced in this chapter will be utilised in Chapter 5 for mutagenesis studies of the *L. pneumophila omp87* gene.

CHAPTER V

Mutagenesis of the

Legionella pneumophila

omp87 gene

5. Introduction

The Omp87 protein is believed to be involved in lipid and protein transport to the outer membrane of the cell. Deletion of the gene in other organisms has resulted in the depletion of lipopolysaccharides and phospholipids from the outer membrane of the bacterial cell, and a corresponding increase and accumulation of these lipids in the inner membrane of the cell (Genevrois *et al* 2003).

Mutagenesis has, for quite some time, been a common and effective method for studying the function of genes. The particular gene of interest can be inactivated by one of several methods, including insertional inactivation or point mutations, and the effect this has on the host organism can be analysed. This often gives a great deal of insight into the most likely function of the gene and its protein product (Salyers and Whitt, 2002).

Following PCR amplification, distribution studies, and cloning of the *L. pneumophila omp87* gene in chapter 4, it was decided that understanding the function of the Omp87 protein would be an important and fundamental addition to the overall knowledge of this novel *L. pneumophila* protein.

5.1 Outline of this chapter

The work undertaken in this chapter describes how the knock-out mutagenesis was performed on the *L. pneumophila omp87* gene, through the insertion of a kanamycin-resistance cassette. The gene is firstly inactivated in the pAOFIA pBluescript construct described in chapter 4. The construct will then be amplified and reintroduced into *Legionella* through electrotransformation.

Through homologous recombination a double cross-over event should take place, resulting in the replacement of the intact chromosomal *omp87* gene with the introduced inactivated *omp87* gene. This should result in a *L. pneumophila* strain without a functional *omp87* gene. By studying the phenotype of this mutant strain, an insight into the function of the *omp87* gene might be obtained.

5.2 Materials and Methods

5.2.1 Inactivation of the *L. pneumophila* FW02/001 *omp87* gene in pBluescript SKII(-)

A non-polar kanamycin resistance gene (*kan*^{R}) isolated from the plasmid pFD666 was used to inactivate the *omp87* gene present in the pAOFIA construct, made in chapter 4, by insertional inactivation. The pFD666 plasmid was kindly donated by Dr. Ryszard Brzezinski, from the University De Sherbrook, Quebec, Canada.

5.2.1.1 PCR amplification of the pFD666 plasmid kanamycin resistance gene

In order to knock-out the *omp87* gene, the *kan*^{R} gene used for the disruption was firstly amplified by PCR from pFD666. The plasmid was supplied as a purified DNA sample, and was therefore firstly electroporated into *E. coli* DH5α cells for propagation (results not shown). The plasmid map of pFD666 is shown in **Figure 5.1**

5.2.1.1.1 Primer design

The primers used to amplify the *kan*^{R} gene were obtained from the publication by Lebeau *et al*, 2004, with the exception that *Bam*HI sites were added to the 5' ends of the primers, to facilitate cloning. The primer sequences, designated KanA and KanB, are shown in **Table 5.1**.

5.2.1.1.2 PCR amplification

The PCR amplification of the *kan*^{R} gene was performed using the Pfu DNA polymerase system (Roche Molecular Biochemicals). The optimised PCR conditions and mastermix components are given in **Table 5.2.**

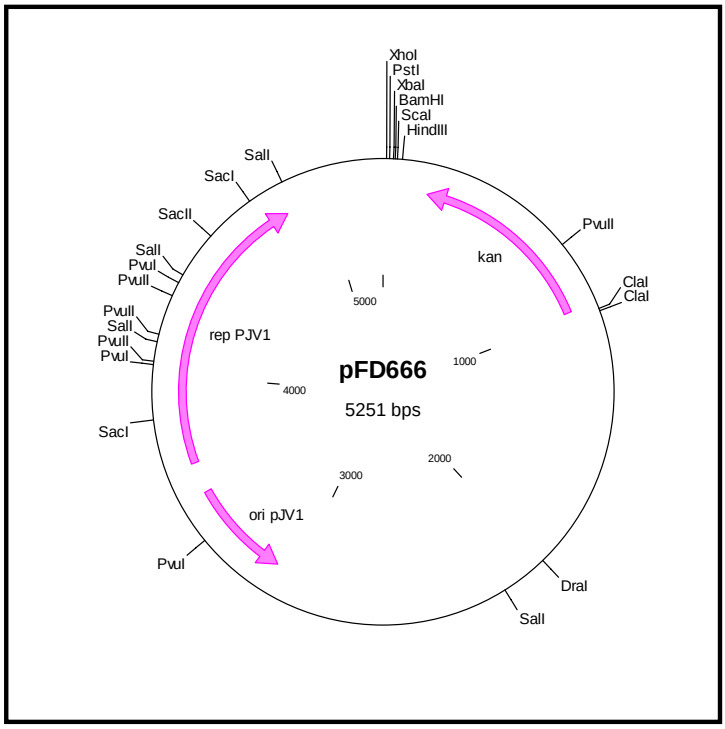


Figure 5.1 A plasmid map of pFD666 which was the source of the *L. pneumophila* compatible kanamycin gene. Arrows indicate the *kan*^{R}, ori pJV1, and the repPJV1 genes.

Table 5.1 Primers designed for amplification of the pFD666 plasmid *Kan*^{R} gene.

Primer	Description	Source/Reference
KanA	5′ TGATGGATCCCAGGGGGGCGGAGCCTATG 3′	Lebeau *et al*, 2004
KanB	5′ GGATGGATCCTACTGCGGCCGCGATCCAAGC 3′	Lebeau *et al*, 2004

* underlined sequence is *Bam*H1 recognition sequence.

Table 5.2 The optimised PCR conditions for amplification of the pFD666 kanamycin gene

PCR Stage	Time / Temp
STAGE 1	
Initial denaturation of DNA template	2 min / 94°C
STAGE 2	
Denaturation of DNA	30s / 94°C
Annealing of primers	30s / 57°C
Elongation	30s / 72°C
Number of cycles	35
STAGE 3	
Final elongation	2 min 30s / 72°C

PCR Master mix components

Reagent	Volume
Milli-Q® water	38 µl
10 x Pfu buffer	5 µl
dNTP's	1 µl
Primer 1 (KanA)	2 µl
Primer 2 (KanB)	2 µl
Pfu polymerase	1 µl
DNA template	1 µl
Total Reaction Volume	**50 µl**

5.2.1.2 Cloning of the *kan*^{R} gene cassette

Following the amplification reaction, the amplified product containing the *kan*^{R} gene was purified using the Promega PCR purification kit (Promega) to remove salts and other impurities that may hinder the activity of enzymes. The purified product was then digested with *Bam*HI to give the PCR product sticky *Bam*HI ends to facilitate cloning. The conditions used for the digestion are described in Chapter 2, Materials and methods.

5.2.1.2.1 Digestion of plasmid pAOFIA

The pAOFIA plasmid, harbouring the *omp87* gene (Chapter 4) was isolated from *E. coli* DH5α using the alkaline lysis method. Digestion of the pAOFIA plasmid was carried out with *Bgl*II. The digestion conditions are described in Chapter 2, Materials and methods.

5.2.1.2.2 DNA Ligation of pAOFIA with the amplified *kan*^{R} gene

The *Bgl*II digested pAOFIA plasmid and the *Bam*HI digested *kan*^{R} gene could be ligated together as *Bgl*II and *Bam*HI enzymes produce compatible sticky ends on the DNA fragments.

Ligations were performed at a ratio of one vector molecule to 2 insert molecules, and one vector molecule to 4 insert molecules, using 10 U of T4 DNA Ligase and T4 DNA Ligase buffer. The reaction mix was made up to 20 µl with sterile Milli-Q® water and incubated at 16°C overnight.

5.2.1.2.3 Electrotransformation

Electrocompetent *E. coli* DH5α cells were transformed by electrotransformation, using a Gene Pulser apparatus (Bio-Rad), set at 25 µF and 1.25 kV, with the Pulse Controller set

at 200 Ω. The ligation mixture was pulsed once at these settings, and immediately after pulsing 1 ml of SOC medium was added. The mixture was then transferred to a 1.5 ml polypropylene tube and incubated at 37°C for 1 hour. One hundred µl and 50 µl aliquots of this suspension were then plated out on LB agar plates containing kanamycin, at a concentration of 50 µg/ml.

5.2.1.2.4 Digestion of plasmids

Colonies obtained following the electrotransformation were re-grown in LB broth, and plasmids were isolated. These were then digested with *Hind*III, at 37°C for 2 hours. Samples were then analysed on a 1% agarose gel. The constructs with the *kan*^{R} in the same orientation as the *omp87* gene were named pAOFIAKanA and the constructs with the *kan*^{R} in the reverse direction were designated pAOFIAKanB.

5.2.1.2.5 PCR amplification of cloned construct pAOFIAKanA/B

Prior to the PCR amplification, the pAOFIAKanA/B clones were linearised by digesting with the enzyme *Eco*RI. The *Eco*RI enzyme cuts the plasmid only once within the multiple cloning site. This process ensured that only DNA which integrated into the bacterial chromosome would confer kanamycin resistance to cells, and not intact pAOFIAKanA/B plasmid carried over from the PCR reaction.

The linearised constructs were then amplified by PCR, using the M13 pBluescript Universal primers. The PCR amplification of the pAOFIAkanA/B constructs was performed using the Pfu DNA polymerase system (Roche Molecular Biochemicals). The conditions used for the reaction were essentially identical to those of the amplification of the *kan* gene from the plasmid pFD666, except that the extension time was extended to 4 min. Clones in both the forward and reverse orientation were amplified, and the resulting PCR products were then used to transform *L. pneumophila* FW02/001.

5.2.1.2.6 Selection of the *L. pneumophila* strain to be used for transformation

Several different strains of *L. pneumophila* were tested for their sensitivity to the antibiotic kanamycin, and hence for their suitability as a host strain for the plasmid constructs pAOFIAKanA/B. Strains were grown on BCYE-α plates containing 50 μg/ml of kanamycin, and BCYE-α plates without kanamycin. Plates were then incubated at 37°C for 48 hours. The strains tested are shown in **Table 5.3**.

Table 5.3 *L. pneumophila* 1 strains tested for kanamycin sensitivity

Strain	Source
L. pneumophila FW02/001	Microbiological Diagnostic Unit (MDU), University of Melbourne, Australia
L. pneumophila ATCC 33152	Microbiological Diagnostic Unit (MDU), University of Melbourne, Australia
L. pneumophila AA100	Dept. of Microbiology and Immunology, University of Kentucky, U.S.A

5.2.2 Natural transformation of *L. pneumophila*

The procedure for the natural transformation of *L. pneumophila* cells was followed according to the method outlined by Stone and Abu Kwaik (1999). Briefly, *L. pneumophila* FW02/001 cultures were grown in 5 ml of Buffered Yeast Extract (BYE) broth at 37°C for 4 days without shaking, in 15 ml capped plastic tubes. Prior to the addition of plasmid DNA, 4.6 ml of BYE broth was removed from the culture,

without disturbing the bacteria settled at the bottom. DNA was then added to the bacterial culture to a final concentration of 40 μg of plasmid DNA per ml, in a final volume of 0.5 ml. The tubes were gently mixed and returned to 37°C for an additional 2 days. One hundred μl of each transformation mixture was then plated out on BCYE-α agar containing 50 μg/ml of kanamycin. Control samples were also used and included the DNA of plasmid pFD666.

5.2.3 Preparation of electrocompetent *L. pneumophila* cells

Electrocompetent *L. pneumophila* cells were prepared using the method described by Cianciotto and Fields (1992). Briefly, 2 plates of *L. pneumophila* FW02/001 grown for 48 hours on BCYE-α plates were harvested and resuspended in 20 ml of sterile distilled H_2O. Cells were then centrifuged at 6000 x *g* for 20 min. The bacterial pellet was then resuspended in 50 ml of ice-cold 10% glycerol in sterile distilled H_2O. The cells were then centrifuged for 25 min at 6000 x *g*. This step was repeated again, with the pellet being resuspended in 50 ml of ice-cold 10% glycerol in sterile distilled H_2O. Cells were then finally resuspended in 500 μl of 10% glycerol and stored at -70°C until used.

5.2.4 Electrotransformation of *L. pneumophila* FW02/001 with pAOFIAKanA/B.

Electrocompetent *L. pneumophila* FW02/001 cells were transformed by electrotransformation, using a Gene Pulser apparatus (Bio-Rad), set at 25 μF and 1.25 kV, with the Pulse Controller set at 200 Ω. The ligation mixture was pulsed once at these settings, and immediately after pulsing 1 ml of BYE broth was added. The mixture was then transferred to a 1.5 ml polypropylene tube and incubated at 37°C for 1 hour. One hundred μl and 50 μl aliquots of this suspension were then plated out on BCYE agar plates containing kanamycin, at a concentration of 50 μg/ml. The rest of the electrotransformation mixture was stored at 4°C for further analysis.
Control samples were also used in the electrotransformation. These consisted of plasmid pFD666 DNA as a positive control, and electrocompetent cells with milli-Q water instead of plasmid DNA as a negative control.

5.2.5 PCR amplification of the *omp87* gene region in transformants.

The PCR was performed on the sample remaining from the electrotransformation of *L. pneumophila.* Firstly, genomic DNA was extracted from the transformation mixture (5.2.5) using the Wizard DNA Purification kit (Promega, U.S.A).

The PCR amplification of the isolated DNA was performed using the AmpliTaq DNA polymerase system (Roche Molecular Biochemicals). The primers used for the reaction included one primer designed to bind to the *L. pneumophila* genomic DNA, around 500 bp upstream of the commencement of the *omp87* gene (CAATTTCGGCACTCGGTACG), and the other primer bound *within* the *kan*^{R} gene (*kan*A or *kan*B). The optimised PCR conditions and mastermix components are given in **Table 5.4.**

5.2.6 DNA sequencing of PCR product from 5.2.6.

The PCR products were separated on an agarose gel and the expected product was excised from the gel prior to DNA sequence determination. The excised gel slice was purified using the QIAquick gel extraction kit (Qiagen, UK). The sequencing reaction mixture, and sequencing reaction cycle conditions are shown in **Table 5.5.**

The sequencing reactions were performed using an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Australia) in a Perkin-Elmer 2400 GeneAmp PCR system. Following the sequencing reaction, the sequencing products were precipitated using ethanol and sodium acetate, according to the manufacturer's instructions.

The DNA sequence determination was carried out by the DNA Sequencing Facility at Monash University, (Clayton campus), Victoria, Australia, using the ABI Prism 373 DNA Sequencer (Perkin-Elmer, Australia).

Table 5.4 The optimised PCR conditions for amplification of transformation mixture

PCR Stage	Time / Temp
STAGE 1	
Initial denaturation of DNA template	1 min / 94 °C
STAGE 2	
Denaturation of DNA	30s / 94 °C
Annealing of primers	30s / 57 °C
Elongation	30s / 72 °C
Number of cycles	35
STAGE 3	
Final elongation	5min / 72 °C

PCR Master mix components

Reagent	Volume
Milli-Q® water	32.5 µl
10 x Taq buffer	5 µl
dNTP's	1 µl
$MgCl_2$	5 µl
Primer 1	2 µl
Primer 2	2 µl
Taq polymerase	0.5 µl
DNA template	2 µl
Total Reaction Volume	**50 µl**

Table 5.5 Sequencing reaction mixture and Sequencing cycle conditions used for DNA sequence determination of the PCR product

Reagent	**Volume**
Ready reaction Premix (2.5)	1 µl
Big Dye Sequencing Buffer (5x)	3.5 µl
Primer (20 ng/ µl)	1 µl
DNA template (PCR product) (8µg/µl)	7.5 µl
MilliQ H_2O	7 µl
Total Volume	**20 µl**
Sequencing reaction cycle conditions	**Time / Temp**
STAGE 1	
Initial denaturation of DNA template	1 min / 96°C
STAGE 2	
Denaturation of DNA	10s / 96°C
Annealing of primers	5s / 50°C
Elongation	4min / 60°C
Number of cycles	25
STAGE 3	
Holding temperature (until purification)	4°C

5.3 Results

5.3.1 Inactivation of the *L. pneumophila omp87* gene in pBluescript SKII-

The construct pAOFIA was used to inactivate the *omp87* gene. This construct was made by inserting the *L. pneumophila omp87* gene into pBluescript SKII- (Chapter 4). A *kan*^{R} gene was isolated from the plasmid pFD666 by PCR and was used to insertionally inactivate the *L. pneumophila omp87* gene.

5.3.1.1 PCR amplification of the pFD666 plasmid kanamycin (*kan*^{R}) gene

The PCR amplification of the kanamycin gene from the plasmid pFD666 was successful, and resulted in a PCR fragment of ~ 1.2 kb. The PCR conditions were optimised, and an annealing temperature of 57°C was found to produce the purest product without compromising product yield (**Figure 5.2)**. The *kan*^{R} gene has a size of 0.8 kb, however the primers used are located outside the gene, to give the product of 1.2 kb. Pfu polymerase was used instead of Taq polymerase for the added benefit of the proofreading activity, to reduce the error rate.

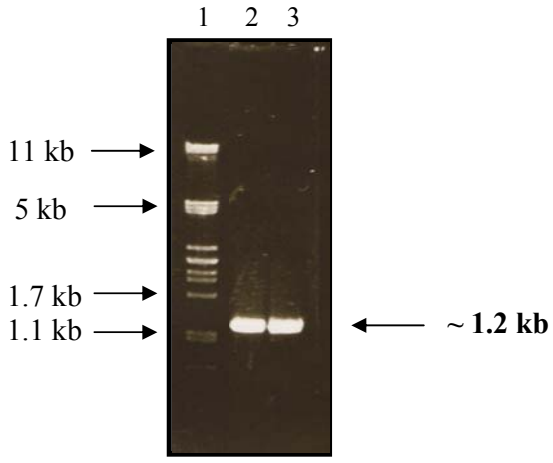


Figure 5.2. PCR amplification of the PFD666 kanamycin gene. Lane: 1, lambda x *Pst*I marker; Lanes 2-3: amplified *kan*^{R} gene.

5.3.1.2 Cloning of the *kan*^{R} gene cassette

Following the amplification and purification of the kanamycin gene product, it was then digested with *Bam*HI to create 'sticky' ends for the ligation process. Similarly, the pAOFIA plasmid, which harbours the cloned *L. pneumophila omp87* gene, was digested with *Bgl*II. A unique *Bgl*II recognition site is present in the pAOFIA plasmid, which is located 449 bp into the *omp87* gene. The enzymes *Bam*HI and *Bgl*II produce compatible sticky ends even though they possess slightly different recognition sequences. The resulting digestion products were ligated together and used to transform *E. coli*.

Colonies resulting from the electrotransformation of *E. coli* DH5α with the ligated pAOFIA and *kan*^{R} gene, were selected on BCYE agar containing 50 μg/ml kanamycin. Plasmid DNA was isolated from the colonies. These were digested with *Hind*III. The digestion products were then separated on an agarose gel, which is shown in **Figure 5.3.** The sizes of inserts expected for each orientation were determined using CloneManager (Clone Manager 6 software, version 6.00, Scientific & Educational Software Inc.), and were as follows:

Forward orientation: 0.95 kb, 1.1 kb and 4.9 kb. This construct was designated pAOFIAKanA **(Figure 5.4a)**.

Reverse orientation: 0.95 kb, 2.3 kb, and 3.7 kb. This construct was designated pAOFIAKanB **(Figure 5.4b)**.

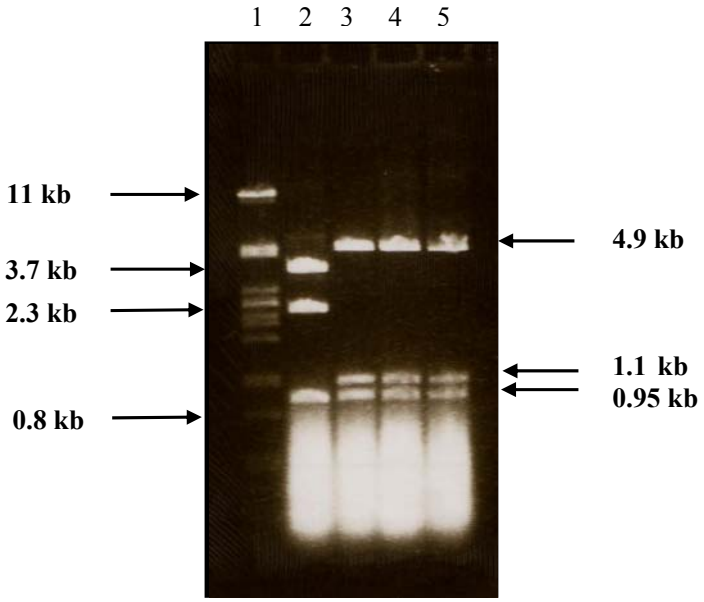


Figure 5.3. Restriction enzyme digests of pAOFIA x *kan*^{R} clones. Lane: 1, lambda x *Pst*I marker; Lane 2-5: *Hind*III digested AOFIAKanA/B clones

From the gel, it is evident that the plasmid analysed in lane number 2 contain the *kan*^{R} gene in the reverse orientation, with product sizes of 3.7, 2.3 and 0.95 kb. The plasmids analysed in lanes 3-5 contain the *kan*^{R} gene in the forward orientation, with product sizes of 4.9, 1.1 and 0.95 kb.

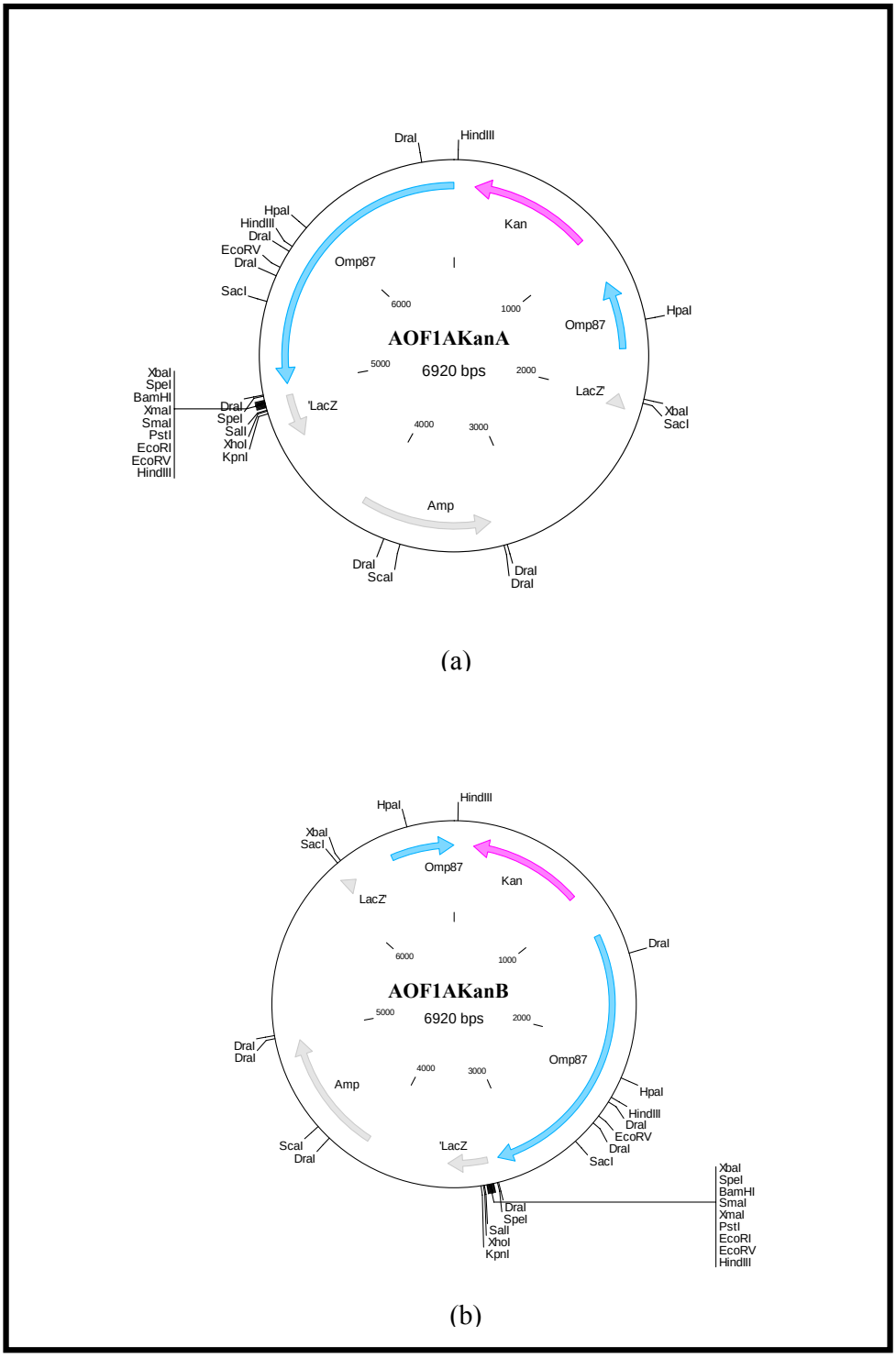


Figure 5.4. Physical map of the pAOFIAKanA/B constructs, with the *kan*^{R} gene in the a) forward orientation, and b) in the reverse orientation. The blue arrow represents the *omp87* gene, whilst the pink arrow represents the *kan*^{R} gene.

5.3.1.3 PCR amplification of cloned construct pAOFIAKanA/B

As the plasmids carrying the pAOFIAKanA/B constructs are not suicide vectors, the plasmids themselves could not be used to transform *L. pneumophila* cells. Therefore a PCR reaction was performed to amplify the inactivated *omp87* gene, and the resulting product was used to transform *L. pneumophila*. Using PCR products to transform *L. pneumophila* is a novel method that has recently been shown to be successful for this organism (personal communication, Emmy De Buck, Rega Institute for Medical Research, Leuven, Belgium). In order to amplify only the region of the disrupted *omp87* gene, the pBluescript Universal M13 primers were used for the PCR reaction. The amplified product was then used to transform the *L. pneumophila* strain. Following transformation, the disrupted *omp87* gene should be introduced into the *L. pneumophila* genome by a double cross-over and replace the functional *omp87* gene, thereby creating a mutant *omp87* gene strain.

The PCR amplification products of the pAOFIAKanA/B constructs can be seen in **Figure 5.5.**

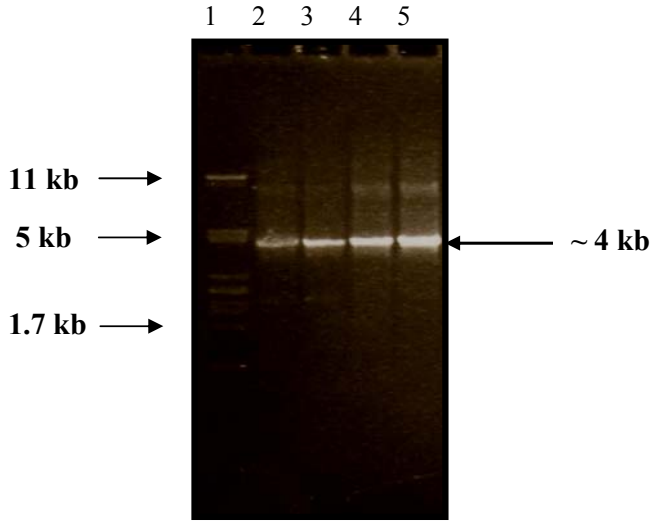


Figure 5.5 PCR amplification of pAOFIAKanA/B constructs. Lane: 1, lambda x *Pst*I marker; Lanes 2-3: PCR amplified pAOFIAKanA clone, Lanes 4-5: PCR amplified pAOFIAKanB clone.

Prior to the transformation of *L. pneumophila*, the PCR products were digested with the restriction enzyme *Eco*RI to linearise the PCR template and prevent this shuttle vector from producing any kanamycin resistant colonies. This enzyme cuts the construct only once, within the plasmid multiple cloning site.

5.3.1.4 Selection of *L. pneumophila* strain for transformation

As the *kan*^{R} gene was used to select for transformants, a *L. pneumophila* strain sensitive to kanamycin had to be used for the transformation experiment. Therefore, different strains of *L. pneumophila* were tested for their sensitivity to kanamycin. This was done by plating out strains on BCYE agar plates containing 50 μg/ml kanamycin.

The results of the *L. pneumophila* sensitivity testing to kanamycin indicated that the *L. pneumophila* AA100 strain was resistant to kanamycin (50 μg/ml), whilst both the FW02/001 and ATCC 33152 strains were sensitive. These results led to the selection of

the kanamycin sensitive strain FW02/001 for use as a host strain for the transformation with the plasmid constructs pAOFIAKanA/B. This strain of *L. pneumophila* was kindly donated by Ms Mary Valcanis at the Microbiological Diagnostic Unit (MDU), University of Melbourne, Australia.

5.3.1.5 Natural transformation of *L. pneumophila* FW02/001

Some strains of *L. pneumophila* are able to take up DNA from their environment, in the process of natural transformation. No colonies were observed following the natural transformation of *L. pneumophila* FW02/001 cells on BCYE-α plates containing kanamycin. All cells grew on plates without kanamycin, indicating that cells remained viable. However, neither test samples nor control samples supported any growth, indicating that cells had not been transformed. The negative result with the positive control sample, plasmid pFD666, was particularly indicative that the natural transformation had not been successful, as we would have expected kanamycin resistant colonies from this control sample.

5.3.1.6 Electrotransformation of *L. pneumophila* with pAOFIAKanA/B

As *L. pneumophila* FW02/001 could not be transformed using natural transformation, electroporation was used.

Following the electrotransformation of *L. pneumophila* with mutant constructs AOFIAKanA/B, the transformation mixtures were plated on BCYE agar plates containing 50μg/ml kanamycin. Following incubation, these plates were then examined for growth. Results of both control and test plates are shown in **Table 5.6**.

Table 5.6. Results of electrotransformation of *L. pneumophila*

SAMPLE	DNA	GROWTH/ NO GROWTH*
+ ve controls	pFD666	Growth
	PCR amplified pAOFIAKanA/B construct	Growth on media without kanamycin
- ve control	H_20	No Growth
Test	PCR amplified pAOFIAKanA construct	No growth
	PCR amplified pAOFIAKanB construct	No growth

* Growth of transformants on BCYE medium supplemented with 50 μg/ml kanamycin, unless otherwise indicated.

From the first positive control plate, where the plasmid pFD666 was electroporated into *L. pneumophila*, the transformation efficiency was calculated. This was shown as an efficiency based on the amount of plasmid DNA used, but also relative to the number of electrocompetent cells present and viable in the starting mixture.

- No. of electrocompetent *L. pneumophila* cells in sample: 1.04×10^{13}
- Number of transformants obtained per ng of plasmid DNA: 7.8×10^{5} cells
- Number of transformants obtained per μg of plasmid DNA: 7.8×10^{8} cells

Therefore the transformation efficiency was:
$\underline{7.8 \times 10^{3} \text{ cells / μg DNA / } 1 \times 10^{8} \text{ cells.}}$

5.3.2 PCR amplification of pAOFIAKanA/B mutants

Due to the fact that no growth was obtained on test plates, a PCR reaction was performed on some of the original electrotransformation mixture to determine whether homologous recombination had taken place between the plasmid-encoded *omp87* gene, and the chromosomally encoded *omp87* gene. A new primer was therefore designed for this reaction.

The primer designed for determining whether the homologous recombination took place, is shown schematically in **Figure 5.6**.

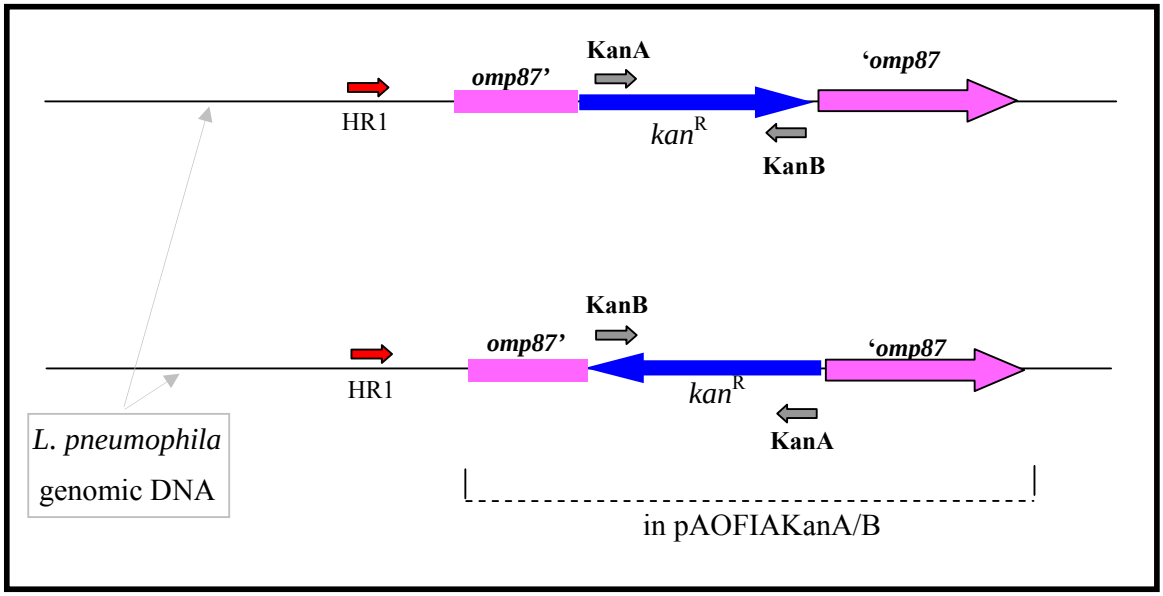


Figure 5.6 Schematic representation of primer design for confirmation of homologous recombination between plasmid DNA and *L. pneumophila* genomic DNA. HR1 (red arrow) represents the new primer HR1; Pink arrow represents *omp87* gene; Blue arrow represents *kan*^{R} gene. KanA and KanB arrows (shaded grey) represent existing primers KanA and KanB. The disrupted *omp87* gene and the *kan*^{R} gene are introduced into the genomic DNA via the plasmids pAOFIAKanA and pAOFIAKanB.

The HR1 primer was designed to bind to *L. pneumophila* genomic DNA, at approximately 500 bp upstream of the commencement of the *omp87* gene. To amplify transformants resulting from transformation with pAOFIAkanA, the primers HR1 and KanB were used. To amplify transformants resulting from transformation with pAOFIAkanB, the primers HR1 and KanA were used.

5.3.2.1 PCR amplification of transformants

In order to determine whether homologous recombination had taken place between the plasmid pAOFIAKanA/B constructs, and the *L. pneumophila* FW02/001 genome, a PCR amplification reaction was performed. Following the electrotransformation of *L. pneumophila* with mutant amplification constructs pAOFIAKanA/B, *L. pneumophila* DNA was extracted and was used as template DNA for a PCR amplification reaction.
The amplification products were then analysed on an agarose gel. These results are shown in **Figure 5.7.**

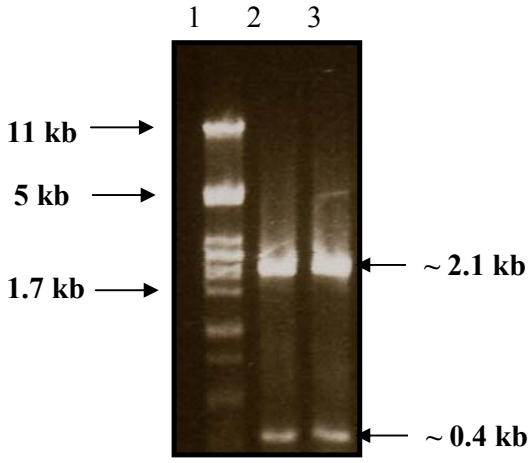


Figure 5.7. PCR amplification of pAOFIAKanA/B constructs. Lane: 1, lambda x *Pst*I marker; Lanes 2: PCR amplified pAOFIAKanA clone, Lane 3: PCR amplified pAOFIAKanB clone.

The 2.1 kb amplified fragment is present, however there was also a smaller amplification product present of ~ 0.4 kb. The 2.1 kb fragment was therefore excised from the gel, purified and sequenced to confirm that it was the region containing both genomic and plasmid DNA.

The sequence obtained from the sequencing reaction is shown schematically in **Figure 5.8**, from the construct pAOFIAKanB. The DNA sequence chromatogram is attached in **Appendix 4**.

The sequencing results showed that the PCR product containing the *kan*^{R} gene had been integrated into the *L. pneumophila* genome by homologous recombination.

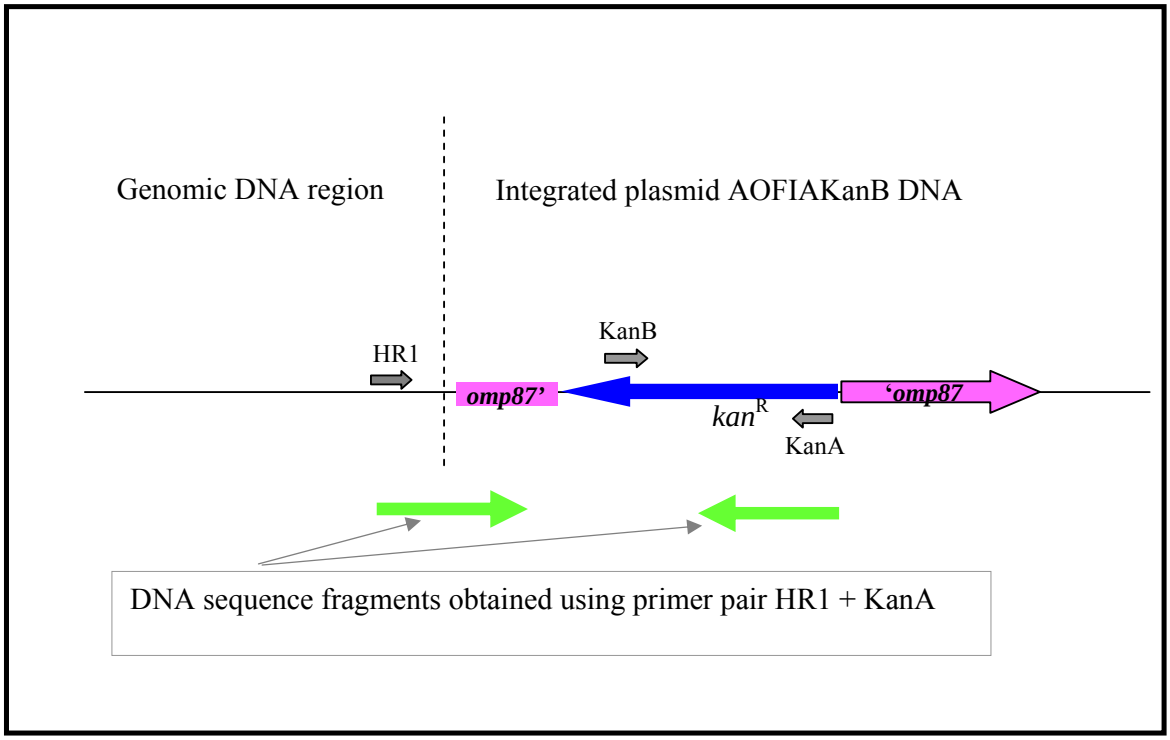


Figure 5.8. Schematic representation of part of the genomic DNA of *L. pneumophila* following homologous recombination with pAOFIAKanB.

The green arrows indicate the area of the DNA sequence analysed using the primers HR1 and KanA. The pink arrows represent the *omp87* gene; the blue arrow represents the *kan*^{R} gene; the grey arrows represent the primers HR1, KanA and KanB.

5.4 Discussion

For numerous years in the 1980s, it was believed that *Legionella* species were not capable of taking up foreign DNA, by either natural or artificial means (Mintz *et al*, 1999). It was not until 1992 when Marra *et al* (1992) discovered that *Legionella* could be transformed with DNA using electroporation. Several years later, Stone and Abu Kwaik (1999) made the interesting discovery that strains of *Legionella pneumophila* which produced type IV pili were naturally competent and able to take up DNA from their surroundings.

Interestingly, the *L. pneumophila* AA100 strain is discussed by Stone and Abu Kwaik (1999) as being one of the strains they determined that was capable of natural transformation. Therefore, this strain was included in this study as the host strain for the mutagenesis of the *omp87* gene, by natural transformation. However, kanamycin sensitivity testing of *L. pneumophila* 1 strains revealed that our laboratory stock of the AA100 strain was resistant to kanamycin. It therefore could not be included in this study, as kanamycin was the antibiotic used for selection with our plasmid. The kanamycin sensitive *L. pneumophila* FW02/001 strain was therefore used as an alternative.

Unfortunately the attempt to naturally transform *L. pneumophila* was not successful. Although the procedure was repeated several times, the control samples failed to produce the expected results. The positive control sample, of pFD666 DNA, if successful, would have provided cells with kanamycin resistance. However no colonies were observed on plates containing kanamycin from any of the samples. This may be because the *L. pneumophila* FW02/001 strain does not possess the receptor molecules necessary for the uptake of DNA through natural transformation. This would not be unusual, as it has been shown that there is often a significant difference in DNA uptake competence, not only between different species, but also *within* species of bacteria (Sikorski *et al*, 2002; Wang *et al*, 2002).

It was then decided that the electroporation of *L. pneumophila*, which has also been well described and extensively carried out, would be used for the transformation. This procedure proved to be straightforward and unproblematic.

In this study, the *omp87* gene of *L. pneumophila* was disrupted through the insertion of a kanamycin-resistance cassette. The *omp87* gene, which had previously been cloned into the plasmid pBluescript SKII- (Chapter 4) was the foundation for the mutagenesis study. In this construct, the *omp87* gene was inactivated through the insertion of a kanamycin gene, resulting in the larger constructs, pAOFIAKanA and pAOFIAKanB. The region of plasmid containing the disrupted *omp87* gene was then amplified by PCR. The resulting PCR product was used to electrotransform *L. pneumophila* FW02/001. No transformants were obtained when the mixture was plated on *Legionella* BCYE-α plates containing kanamycin.

Several other factors may have caused the lack of kanamycin resistant colonies after *L. pneumophila* was transformed with pAOFIAkanB. Firstly, there was a possibility that the *L. pneumophila* cells had not been permissive for the electrotransformation, and had not actually taken up any of the foreign DNA. This may have been due to the particular strain used, or that the procedure used for preparing the electrocompetent cells had not been successful.

Secondly, the possibility would also exist that homologous recombination had not occurred as expected, and the double cross-over event had not taken place. This would prevent the integration of the kan^R gene into the genome and therefore none of the organisms would possess resistance to the antibiotic. This was an important consideration as the cloned *omp87* gene was obtained from a different *L. pneumophila* strain (AA100) than the strain used for the transformation experiments (FW02/001).

This change in strain was necessary, as the AA100 strain was already kanamycin resistant. These strains may have possessed subtle differences in DNA sequences which could be sufficient to inhibit a successful double cross-over recombination event.

Thirdly, the inactivation of the *omp87* gene could have been lethal to the *L. pneumophila* cells.

The inclusion of control samples was one of the key components to elucidate the cause for the lack of kanamycin resistant colonies. In addition to the negative control samples, a positive control sample was included, consisting of plasmid pFD666 DNA. This is the plasmid from which the kanamycin gene was amplified.

Kanamycin-resistant colonies were obtained using this control sample, therefore indicating that the *L. pneumophila* FW02/001 strain used was indeed capable of taking up foreign DNA,

The transformation efficiency of the *L. pneumophila* with this plasmid was determined, and was found to be 7.8 x 10^3 cells / μg DNA / 1x10^8 cells. This figure falls within the range of the transformation efficiencies observed for other organisms with similar sized plasmids, and similar starting numbers of electrocompetent cells (i.e. ~ 10^8 cells). Certain organisms however, such as some strains of *E. coli*, can achieve efficiencies of up to 10^7 to 10^8 cells / μg DNA (Calvin and Hanawalt, 1988). Some strains of *Campylobacter jejuni* were also found to achieve high efficiency values of up to 1.6 x 10^5 cells / μg DNA (Miller *et al*, 1988).

In order to confirm that homologous recombination had taken place, a PCR amplification reaction was performed. The novel approach described by Burns *et al* (2000) was used, whereby a PCR product would only be obtained if the double cross-over event had occurred. This was achieved by the effective and unique design of primers, whereby one primer was designed in the genome of the organism, whilst the other was designed on the integrated *kan*^{R} gene. Integration of part of the plasmid into the genome could only occur by a double cross-over event as the plasmid had been linearised by restriction digestion prior to the PCR amplification and electrotransformation. Therefore, no intact plasmid could have been introduced into the *L. pneumophila*, which might have conferred kanamycin resistance to cells. Therefore if a PCR product was obtained, we could conclude that homologous recombination had taken place.

As the PCR reaction was successful, and a fragment of the expected size was obtained, the final confirmatory stage was to determine the DNA sequence of this fragment, and to observe whether the sequence obtained correlated with that expected if the double cross-over event had taken place.

As anticipated, the DNA sequence derived from the transformed cells corresponded to the expected sequence. This included part of the *L. pneumophila* genomic DNA sequence, part of the *omp87* gene sequence, and part of the sequence of the kanamycin resistance cassette. As mentioned, this was already anticipated due to the previous PCR amplification reaction, which gave a PCR product size indicative of a successful double cross-over event.

From this study, we were able to conclude that the inactivation of the *omp87* gene in *L. pneumophila* FW02/001 resulted in non-viable cells. We demonstrated that a double cross-over event occurred within the *L. pneumophila* genome between the

chromosomally encoded *omp87* gene, and a mutated, plasmid encoded copy of the gene, and that this must have been lethal to the cells.

Further investigations are needed to definitively conclude that this lethality was due to the fact that the *omp87* gene of *L. pneumophila* was an essential gene. The effect of the mutation on genes downstream of the mutated *omp87* gene would need to be analysed to confirm that the mutation did not induce any polar effects, which may also have given rise to the lethality.

Further work is also needed to increase our understanding of the function of the Omp87 protein in *L. pneumophila*. This analysis becomes more difficult if mutagenesis studies cannot be relied upon for providing insight into the role of this protein in the biology of the cell. In the case of lethal mutations, it then becomes necessary to analyse the function of the gene by means of inducible promoters or genes, cloned upstream of the gene of interest. The expression of these genes can then be regulated to switch on and off. In this way, the effect of little or no expression of the gene can be analysed without necessarily being detrimental to the cell.

Several mutagenesis studies have been performed on genes belonging to the Omp family in other organisms, and these have had mixed results. *Neisseria meningitidis* is one such strain. Genevrois *et al* (2003) were successfully able to control the expression of the *N. meningitidis omp85* gene by the use of a *tac* promoter. This promoter allowed expression of the gene to be switched on and off, allowing for the analysis of the gene, without compromising the cell's viability. They found that depletion of the Omp85 protein resulted in an accumulation of lipopolysaccharide and phospholipids in the inner membrane of the cell, and their disappearance from the outer membrane. Its function was therefore linked with the transport of LPS to the bacterial outer membrane.

In contrast to this, Bos *et al* (2004) believe that the presence of Omp85 homologues in organisms which do not contain LPS is more indicative of a more minor role in LPS transport, and a more critical role in the transport of Omps.

The Omp85 protein of *E. coli*, also known as YaeT, is believed to play the same role, of Omp transport, in *E. coli*. Werner and Misra (2005) recently examined the outer membrane of *E. coli*, and found that cells which were depleted of the Omp85 protein were severely affected, and showed an accumulation of Omps in the periplasm of the cell. It was therefore speculated that the protein is involved in inserting the soluble protein intermediates from the periplasm into the outer membrane.

Before the discovery that *Legionella* spp. are able to take up DNA both naturally and artificially, the study of *Legionella* spp. pathogenicity and virulence was very limited and time-consuming. Since then however, there has been a rapid expanse of knowledge and understanding into *Legionella* spp. and their pathogenic mechanisms and cellular functions. However studying essential genes still remains problematic.

CHAPTER VI

Legionella pneumophila Omp 87:

Protein expression

and

analysis

6. Introduction

Recombinant protein expression and purification is a routinely used laboratory approach for analysing proteins, or peptides. The purified proteins can be used to identify their functional role, structure, antigenic properties or potential as vaccine candidates.

Protein expression in high level expression vectors, such as pRSET, is a common and effective means of producing high amounts of a particular protein. The pRSET Expression vector (Invitrogen) which is a derivative of the pBluescript plasmid, is specifically designed for high level expression of prokaryotic proteins, and is controlled by the bacteriophage T7 promoter (www.Invitrogen.com). This T7 promoter system was originally described by Studier *et al* (1986) and functions on the principle that induction of the promoter lacUV5, by IPTG, leads to cellular expression of T7 DNA polymerase, which in turn leads to the expression of the cloned gene of interest. The host cells used with the system, *E. coli* BL21(DE3) pLysS, possess a chromosomal copy of the T7 DNA polymerase gene. These cells also possess an additional plasmid, the pLysS plasmid, which encodes a T7 lysozyme gene, to repress and prevent any basal expression of cloned target genes. In this way, any 'leaky' expression of the target gene can be controlled and minimised so that it is only expressed when the inducer IPTG is added to growth medium.

Importantly, the pRSET vector contains a poly-histidine (6xHis) tag, so that the protein can be later purified using Ni-NTA Sepharose-based resins. The histidine tag binds reversibly to the Ni^{2+} residues in the sepharose resin, to allow for extraction and purification of the expressed protein from the total protein content of the cell. The histidine-tagged protein can then be eluted from the sepharose column by competitive binding with imidazole, or by using buffers with a reduced pH (Bollag *et al*, 1996).

The pRSET vectors are available in three different reading frames (pRSETA, B and C), relative to the multiple cloning site sequence to facilitate cloning of proteins in frame with the 6 Histidine residues. In this study, the vector pRSET A was used.

6.1 Outline of this chapter

This chapter describes the cloning, expression, purification and analysis of the Omp87 protein. The expression vector pRSETA was used for expression of the Omp87 protein, and four truncates of varying sizes were designed, through the use of different PCR primers. The constructs were then sequenced, and immunoblotting was performed with anti-histidine antiserum. The protein truncates were then expressed and purified using Ni-NTA sepharose resin (Qiagen) packed columns and gravity flow chromatography.
The purified protein was then analysed by SDS-PAGE and Western blotting, using human, anti-*Legionella* antiserum.

6.2 Materials and Methods

6.2.1 Cloning of the *omp87* gene into pRSETA

6.2.1.1 Design and PCR amplification of truncated *omp87* gene fragments

The *L. pneumophila* serogroup 1 AA100 strain was used to PCR amplify four different fragments of the *omp87* gene. The primer sequences of each of the fragments are shown in **Table 6.1**. The largest construct, construct 1, contains its own set of forward (C1A) and reverse (C1B) primers. The other 3 constructs all have different forward primers (C2A, C3A and C4A) but share the same reverse primer (C234B), with incorporated His-tag (underlined). The PCR amplification of the truncated *omp87* gene was performed using the Pfu DNA polymerase system (Roche Molecular Biochemicals). The optimised PCR conditions and mastermix components are given in **Table 6.2.** Following the PCR amplification reaction, 3 identical samples were combined to increase the concentration of DNA available for cloning. The combined samples were then purified using the Wizard PCRprep purification kit (Promega).

6.2.1.2 Isolation and digestion of plasmid pRSETA

A miniprep isolation of plasmid pRSET was performed by the alkaline lysis method (Ausubel *et al*, 2005). A freshly grown culture of *E. coli* BL21 cells harbouring the plasmid were used for the extraction.

The purified plasmid was then digested with the restriction enzymes *Pst*I and *Eco*RI, according to the method described in chapter 2 (Materials and methods). The ability of each enzyme to cut the plasmid was tested by single digestions, followed by a double digestion with both enzymes under identical conditions. Digestions were performed at 37°C for 2 hours. Following the digestion, the restriction enzymes were inactivated by heating at 65°C for 15 min.

Table 6.1. Primers designed for amplifying fragments of the *omp87* gene

Primer	Description
C1A	3' TTAACTGCAGAAGTGCGCGTAGTGCTGGAC 5'
C2A	3' TCAACTGCAGTAGAGTTAACGGATTACAAAG 5'
C3A	3' TGGACTGCAGGGAGAAGGAGCTGAAACAGGCCTAC 5'
C4A	3' AATCCTGCAGTGCAATAGGGTTAGCTTTAG 5'
C1B	3' ACCTGAATTCACCTGAGGAAAGAGCAAATTGGAAGA 5'
C234B	3' TTAGAATTGATGATGATGATGATGATGACCTGAGGA--AAGAG CAAATTGGAAGA 5'

* Underlined region outlines position of Histidine tag

Table 6.2 The optimised PCR conditions for amplification of *L. pneumophila omp87* truncated gene constructs

PCR Stage	Time / Temp
STAGE 1	
Initial denaturation of DNA template	1 min / 94°C
STAGE 2	
Denaturation of DNA	30s / 94°C
Annealing of primers	30s / 49°C
Elongation	5min* / 72°C
Number of cycles	35
* Based on construct 1 (i.e. shorter elongation time for smaller constructs)	
STAGE 3	
Final elongation	5min / 72°C

PCR Master mix components

Reagent	Volume
Milli-Q water	38 µl
10 x PCR buffer	5 µl
dNTP's	1 µl
Primer 1	2 µl
Primer 2	2 µl
Pfu polymerase	1 µl
DNA template	1 µl
Total Reaction Volume	**50 µl**

6.2.1.3 Digestion of Truncated *omp87* gene fragments

Following the purification of the *omp87* PCR products, the samples were all digested with the restriction enzymes *Pst*I and *Eco*RI, prior to ligation with the plasmid pRSETA. Digestions were performed at 37°C for 2 hours. Following the digestion, the restriction enzymes were inactivated by heating at 65°C for 15 min.

6.2.1.4 Ligation of *omp87* gene PCR products with plasmid pRSETA

Ligations were performed in the ratio of one vector molecule to 2 insert molecules, and one vector molecule to 4 insert molecules. The ligations were performed with 10 U of T4 DNA Ligase (Boehringer Mannheim) and T4 DNA Ligase buffer. The reaction mix was made up to 20 µl with sterile Milli-Q® water and incubated at 16°C overnight. The mixture was then used for transformation of *E. coli* BL21 cells.

6.2.1.5 Electrotransformation

Electrocompetent *E. coli* BL21 cells were transformed by electrotransformation, using a Gene Pulser apparatus (Gene Pulsar^{TM}, Bio-Rad), set at 25 µF and 1.25 kV, with the Pulse Controller set at 200 Ω. The ligation mixture was pulsed once at these settings, and immediately after pulsing 1 ml of SOC medium was added. The mixture was then transferred to a 1.5 ml polypropylene tube and incubated at 37°C for 1 hour. One hundred µl of this suspension was then plated out on LB agar plates, containing 100 µg/ml ampicillin.

6.2.2 Screening of recombinant plasmids

Colonies resulting from the electrotransformation procedure were re-grown in LB broth containing 50 µg/ml ampicillin for 24 hours at 37°C. Plasmids were then isolated and

digested with the restriction enzymes *PstI* and *Eco*RI, under the same conditions detailed in section 6.2.1.2 for the isolation and digestion of plasmid pRSETA.

6.2.3 DNA sequencing of the recombinant pRSET constructs

The pRSET T7 Promoter primer (5' TAATACGACTCACTATAGGG 3'), and reverse primer (5' TATGGCTAGCATGACTGGT 3') whose sequences were obtained from the Invitrogen pRSET technical manual (www.Invitrogen.com) were used for the DNA sequencing reaction of the recombinant *omp87* fragments. The sequencing reactions were performed using an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Australia) in a Perkin-Elmer 2400 GeneAmp PCR system. The sequencing reaction mixture and sequencing reaction cycle conditions can be seen in **Table 6.3**. Following the sequencing reaction, the sequencing products were precipitated using ethanol and sodium acetate, according to the manufacturer's instructions.

The DNA sequence determination was carried out by the Micromon DNA Sequencing Facility at Monash University, (Clayton campus), Victoria, Australia, using the ABI Prism 373 DNA Sequencer (Perkin-Elmer, Australia).

6.2.4 Expression of pRSET constructs in *E. coli* BL21

6.2.4.1 Growth and induction of pRSET plasmid constructs

In order to determine the optimal induction time for each pRSET *omp87* construct, the following study was performed. Briefly, 2 ml of LB broth containing 50 µg/ml of ampicillin was inoculated with each construct, and was grown overnight at 37°C with shaking. The following day, 25 ml of broth was inoculated with the overnight culture. The culture was then grown at 37°C with vigorous shaking to an OD_{600} (l = 1cm) of between 0.4 - 0.6. One ml of culture was then removed as the time zero sample. The inducer IPTG was then added to the broth to a final concentration of 1 mM, and the

culture was grown for several more hours, with a 1 ml sample of the broth being taken at hourly intervals.

In addition to the optimal time for IPTG induction of the recombinant pRSET protein constructs, several other parameters were also investigated. These included the concentration of IPTG used for induction, and the temperature cultures should be grown at during induction. In the case of the optimal IPTG concentration to be used, several concentrations were tested. These included 0.2 mM, 0.5 mM, 1 mM, 5 mM and 10 mM. These final concentrations were all used to induce cultures which had reached an OD_{600} of 0.4. Cultures were then induced for 5 hours, at 37°C.

For the investigation into the optimal induction temperature to be used, the temperatures of 28°C, 32°C and 37°C were tested. These cultures were all grown to an OD_{600} of 0.4, whereby they were then induced with 1 mM IPTG. The cultures were then incubated for 5 hours at one of the above mentioned temperatures.

The samples were then analysed by SDS-PAGE analysis.

6.2.4.2 Determination of truncated Omp87 protein solubility

In order to determine whether the expressed Omp87 protein was soluble or insoluble, the cells containing the induced pRSET constructs were boiled in a small volume of SDS-PAGE loading buffer, containing the reducing agent β-mercaptoethanol. The samples were then centrifuged, and both the supernatant and pellet were analysed by SDS-PAGE to determine which fraction contained the recombinant protein.

The program devised by The School of Chemical Engineering and Materials Science at the University of Oklahoma (http://biotech.ou.edu/) was also used for an estimate of the solubility of the proteins. The protein sequences were entered into the program, and the predicted percentage of the protein's solubility or insolubility was determined.

Table 6.3. Sequencing reaction mixture and Sequencing cycle conditions used for DNA sequence determination of recombinant pRSET protein constructs

Reagent	**Volume**
Ready reaction Premix (2.5x)	1 µl
Big Dye Sequencing Buffer (5x)	3.5 µl
Primer (20 ng/µl)	1 µl
DNA template (PCR product) (8 ng/µl)	7.5 µl
MilliQ H_2O	7 µl
Final volume	**20 µl**

Sequencing reaction cycle conditions	**Time / Temp**
STAGE 1	
Initial denaturation of DNA template	1 min / 96°C
STAGE 2	
Denaturation of DNA	10s / 96°C
Annealing of primers	5s / 50°C
Elongation	4min / 60°C
Number of cycles	25
STAGE 3	
Holding temperature (until purification)	4°C

6.2.4.3 Western blotting of Omp87 recombinant protein with anti-His antiserum

To confirm that the histidine tag was correctly expressed from the recombinant Omp87 pRSET constructs, immunoblotting was performed using mouse anti-his antiserum. The primary antiserum was used at a dilution of 1/3000. The secondary antibody, a goat, anti-mouse IgG was used at 1/5000. Separation of proteins by SDS-PAGE was firstly performed. An aliquot of the sample taken prior to column purification (pre-column sample), together with purified construct 3 and purified construct 4 were analysed. The SDS-PAGE gel was then used for the immunoblotting procedure. The details of this procedure are described in Chapter 2, Materials and methods.

6.2.4.4 Purification of truncated Omp87 proteins by gravity-flow chromatography

6.2.4.4.1 Pre-treatment of samples for column purification

Prior to the purification of the expressed protein from the total cell protein content by sepharose column chromatography, solubilisation of the expressed protein was required, so as to enable the effective unfolding and binding of the protein to the Ni^{2+} charged sepharose. This procedure was derived from that of the purification of Omp85 from *Neisseria meningitidis* (Dr. George Moutafis, Pfizer Australia, personal communication).

Briefly, following the induction of the *E. coli* BL21 cells for 4 hours with 1 mM IPTG, the cells were harvested and pelleted by centrifugation at 5000 x *g* for 15 min. The supernatant was removed, and the pellet was resuspended in 5 ml of wash buffer (50 mM Na_2PO_4, 300 mM NaCl, 20 mM imidazole). The protease inhibitors Leupeptin (1μg/ml) and Benzamidine (1nM) were added to the sample. Ten mg of lysozyme was also added and the samples were incubated at 37°C for 30 min. The samples were then frozen at – 70°C for 30 min. A series of freeze-thawing cycles were then performed

using a dry ice/ethanol bath, and a 37°C water bath. Five U of DNase was then added to remove DNA and therefore reduce the viscosity of the sample. The sample was then centrifuged at 1000 x *g* for 15 min at room temperature to pellet the insoluble inclusion bodies. Ten ml of 8 M Urea (in wash buffer) was then added to samples to solubilise inclusion bodies, and the samples were left shaking gently at room temperature for 1.5 hours. A 1 ml aliquot of the sample was collected at this stage for analysis (pre-column sample).

6.2.4.4.2 Purification by gravity flow chromatography

Following pre-treatment, the samples were then added to a 5 ml sepharose-filled vertical column which had been pre-equilibrated with 10 ml of 8 M Urea (in wash buffer). The flow through from the column was collected for analysis (flow through sample). The column was then washed with 20 ml of progressively lower concentrations of urea through the column (8 M, 6 M, 5M, 4 M, 3 M, 2 M). The recombinant protein was then eluted from the column by washing with 20 ml of elution buffer (2 M Urea, 50 mM Na_2PO_4, 300 mM NaCl, 500 mM imidazole). One ml aliquots were collected during the elution process. The fractions were then analysed with a simplified Bradford assay to determine which fractions contain the recombinant protein. In this assay, 20 µl of the sample were mixed with 200 µl of Bradford reagent (0.01% Coomassie Brilliant G-250 dye, 5% 95% Ethanol, and 10% Phosphoric Acid (85%), in dH_20). The samples were then observed for colour development, which was relative to the amount of protein present. The 4 or 5 aliquots generating the deepest colour development were then pooled together and analysed by SDS-PAGE.

6.2.4.5 SDS-PAGE Analysis of proteins

Protein samples were analysed by SDS-PAGE, according to the method described in Chapter 2, Materials and methods.

6.2.4.6 Immunoblotting of recombinant proteins with anti-*Legionella* antiserum

Following the purification of the recombinant pRSET constructs 3 and 4, immunoblotting was performed to determine whether the recombinant proteins were recognised by anti-*L. pneumophila* antibodies following their expression in the *E. coli* BL21 expression system. Western blotting was therefore carried out as outlined in Chapter 2, Materials and methods.

Approximately 25 µg of each of the recombinant proteins was firstly subjected to SDS-PAGE analysis. A whole cell lysate of *L. pneumophila* AA100 was also prepared and included as the positive control. Immunoblotting was then performed using a batch of human anti-*Legionella* antiserum that was kindly donated by the Serology department at the Victorian Infectious Disease Reference Laboratory (VIDRL). The primary antiserum was used at a dilution of 1/3500. The secondary antiserum, anti-human IgG was then used at a dilution of 1/3000.

6.3 Results

6.3.1 Design of primers to amplify truncated *omp87* gene fragments

Different primer sets were designed to amplify different fragments of the *omp87* gene. These truncates varied in size from 2.37 kb, to 1.4 kb, and were designed to be progressively smaller and truncated from 5' end of the gene (the proteins N-terminal region).

The largest amplicon, fragment 1, had a forward primer designed to include 5 additional amino acids (15 bp) upstream of the start codon of the *omp87* gene, to ensure the expression of the complete gene and include the entire secretion signal sequence of the protein. The sizes of each of the fragments were as follows:

Fragment 1: 2376 bp

Fragment 2: 2223 bp

Fragment 3: 1911 bp

Fragment 4: 1392 bp

6.3.2 PCR amplification of truncated *omp87* gene fragments.

Truncated *omp87* gene fragments were created by PCR amplification of the *L. pneumophila omp87* gene using different sets of primers **(Table 6.1)**. The amplification of the constructs was performed successfully, however the PCR product yield for Fragment 2 was low, despite optimisation of the PCR conditions. In order to increase this yield, it was decided to use amplified fragment 1 DNA as a template for the amplification of the slightly smaller fragment 2. This provided a higher amount of template DNA, as it had already been amplified by PCR. The original PCR amplification of fragment 2 can be seen in **Figure 6.1**, and the subsequent PCR reaction, using fragment 1 DNA as a template for the reaction, can be seen, along with all other fragments, in **Figure 6.2.** The yield of fragment 2 has increased, however there is a small amount of non-specific product of around 5 kb.

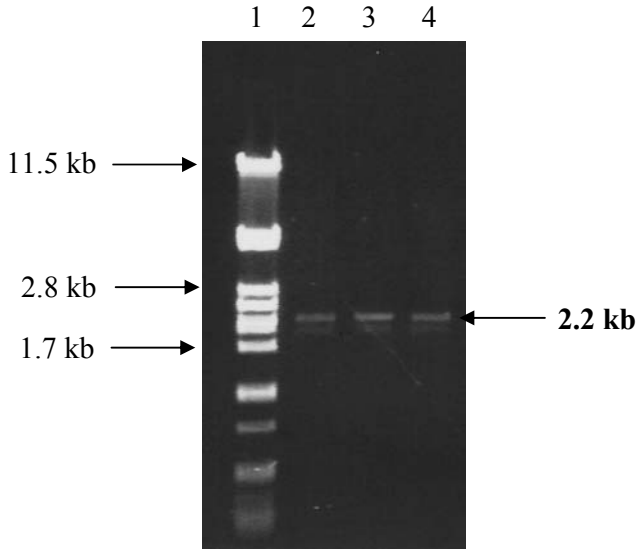


Figure 6.1 PCR amplification of *omp87* fragment 2, with an amplified product size of ~ 2.2 kb. Lane: 1, lambda x *Pst*I marker; Lanes 2-4, PCR amplification product of *omp87* fragment 2, of 2.2 kb.

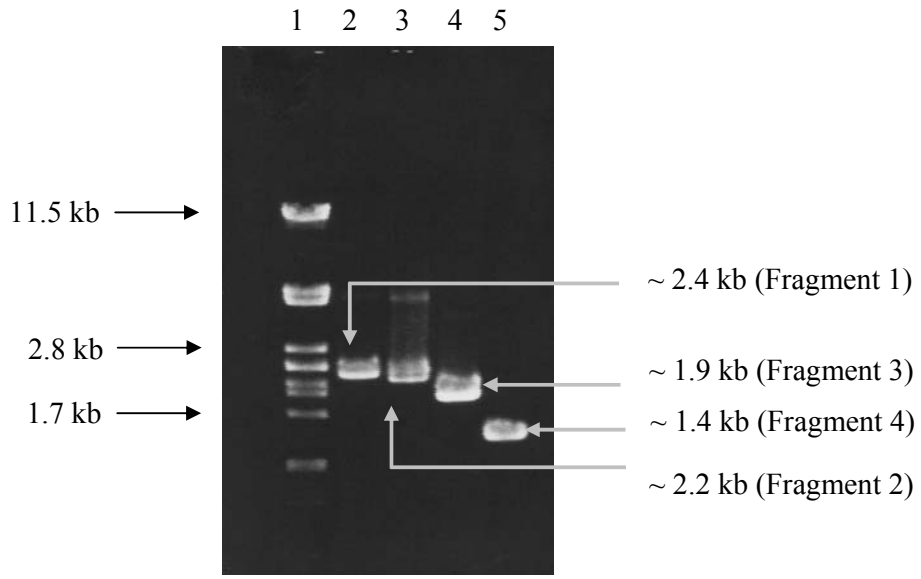


Figure 6.2 PCR amplification of *omp87* fragments 1-4. Lane: 1, lambda x *Pst*I marker; Lanes 2-5, PCR amplification product of Omp87 truncates 1-4, of sizes 2.4 kb, 2.2 kb, 1.9 kb; and 1.4 kb, respectively.

6.3.3 Isolation and restriction digestion of plasmid pRSET

The plasmid to be used for the expression of the *omp87* fragments, pRSET, was successfully isolated from a fresh culture of *E. coli* BL21 cells by the alkaline lysis method. A schematic diagram of this plasmid can be seen in **Figure 6.3**.

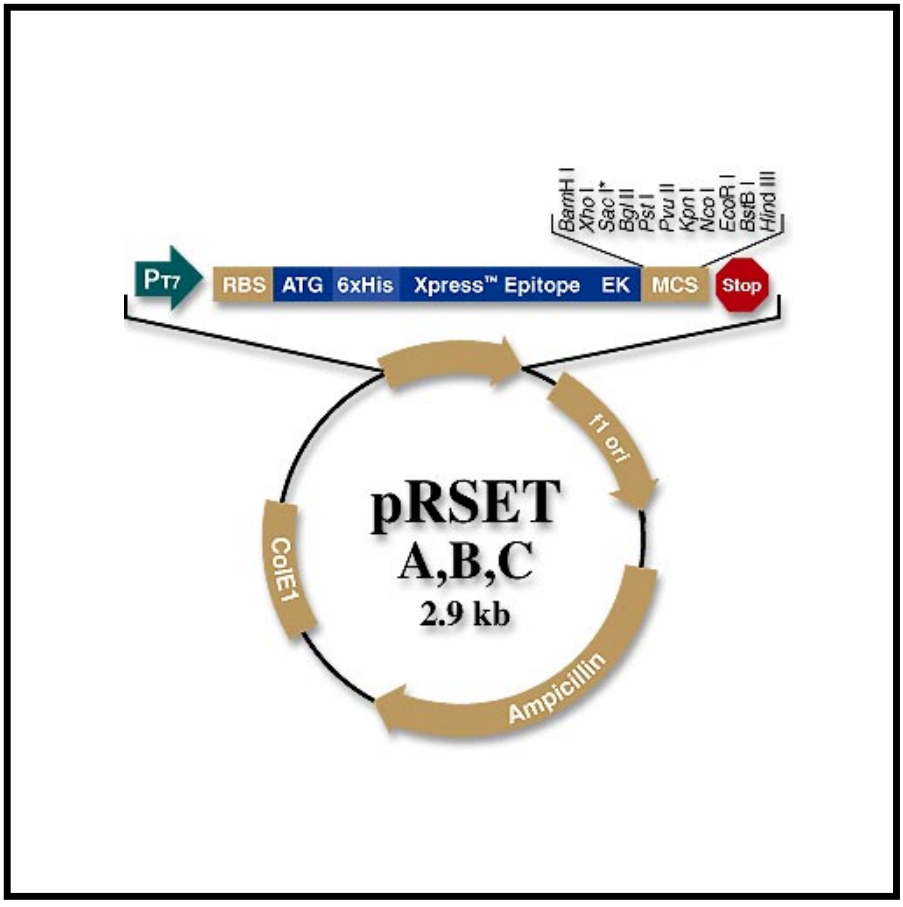


Figure 6.3. Physical map of the plasmid pRSET, displaying the major plasmid features (www.Invitrogen.com). These include the T7 Promoter gene (P_{T7}), the 6 x Histidine tag (6 x His), the Ribosome binding site (RBS), the Multiple cloning site (MCS), the *E. coli* origin of replication (ColE1), the Phage 1 origin of replication (f1 ori) and the ampicillin resistance gene.

In order to insert the PCR fragments into the pRSET plasmid, single and double digestions were performed, using the enzymes *Pst*I and *Eco*RI to ensure complete digestion by both enzymes. The results of the digestions are shown in **Figure 6.4**.

6.3.4 Electrotransformation of pRSET constructs into *E. coli* BL21

Following the digestion of the *omp87* fragments and the pRSET plasmid, ligations were performed. The constructs we expected to obtain following the ligation can be represented schematically, and the physical maps for all four constructs are shown in **Figures 6.5 - 6.8.** The insert sizes expected for each of the clones were as follows:

pRSET Construct 1 : 2.9 kb + 2.3 kb; pRSET Construct 2 : 2.9 kb + 2.2 kb; pRSET Construct 3 : 2.9 kb + 1.9 kb; pRSET Construct 4 : 2.9 kb + 1.4 kb.

The ligated constructs were then used to transform *E. coli* BL21 cells via electrotransformation. The colonies resulting from the electrotransformation were grown in broth, and plasmids were isolated and digested with the restriction enzymes *Pst*I and *Eco*RI. Results of the digestions can be seen in **Figure 6.9.**

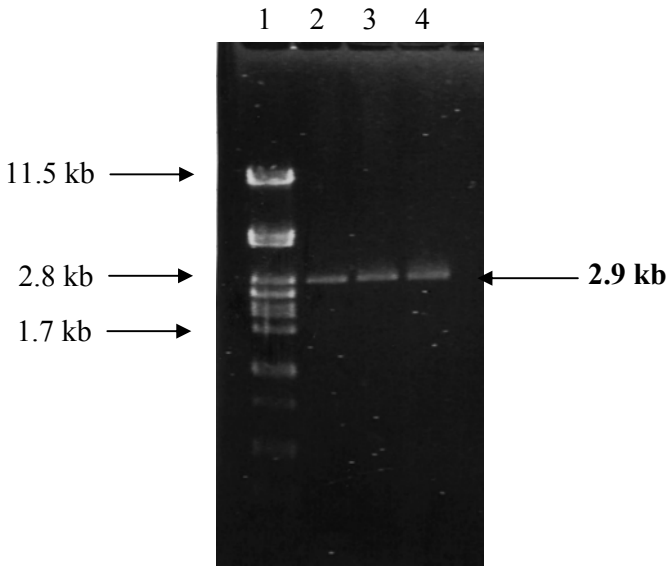


Figure 6.4 Restriction digestion of plasmid pRSETA. Lane: 1, lambda x *Pst*I marker; Lane 2, pRSET x *Pst*I; Lane 3: pRSET x *Eco*RI; Lane 4: pRSET x *Pst*I, *Eco*RI.

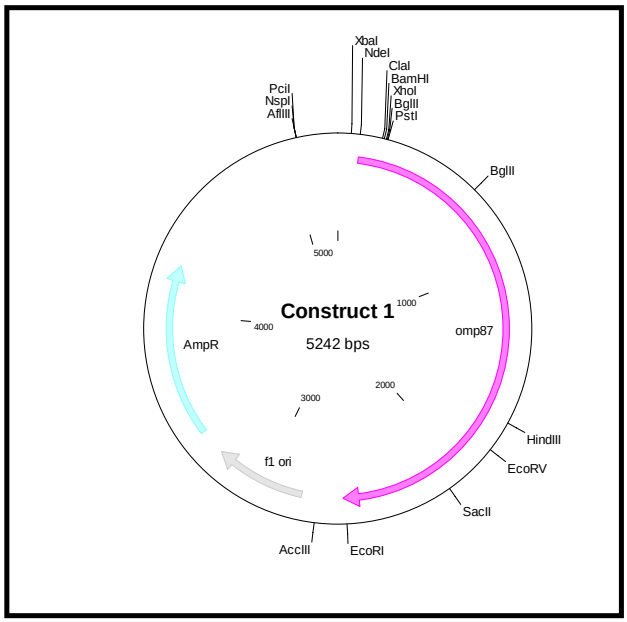


Figure 6.5 – Physical map of the plasmid pRSET, Construct 1, containing the largest of the four *omp87* gene inserts of 2.5 kb. The *omp87* gene is represented by the pink shaded arrow. The *amp*^{R} gene is represented by the light blue shaded arrow.

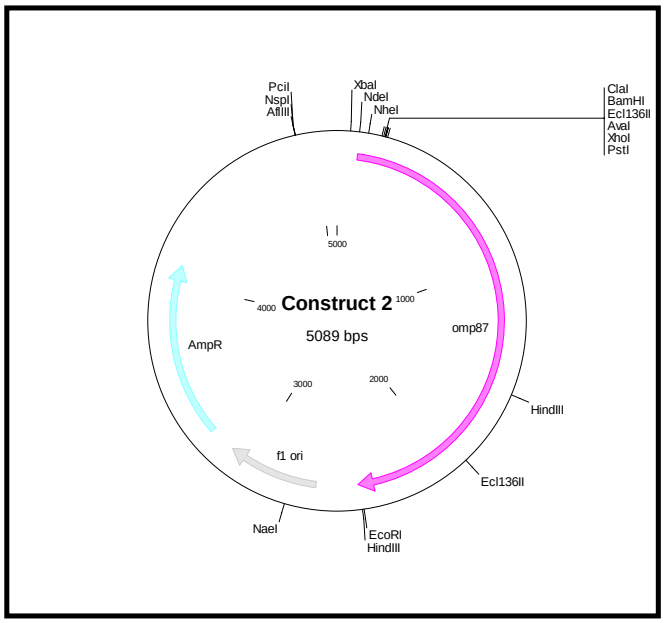


Figure 6.6 – Physical map of the plasmid pRSET, Construct 2, containing an *omp* insert of 2.3 kb. The *omp87* gene is represented by the pink shaded arrow. The *amp*^{R} gene is represented by the light blue shaded arrow.

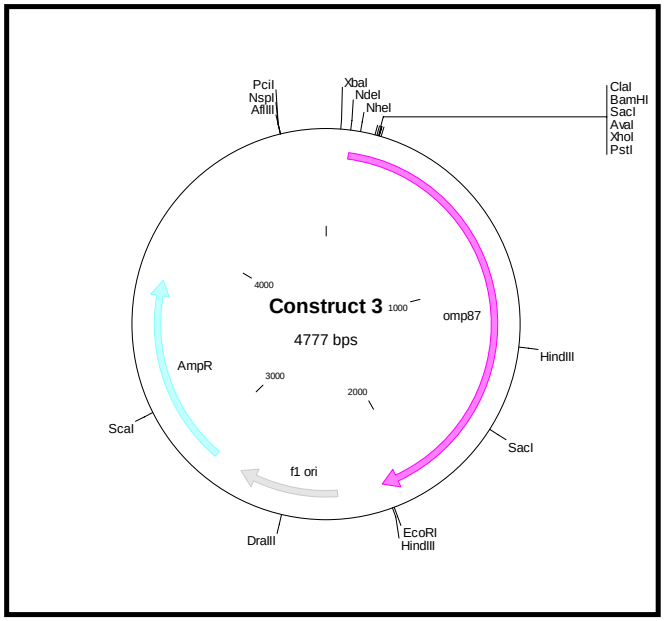


Figure 6.7 – Physical map of the plasmid pRSET, Construct 3, containing an *omp* insert of 2.0 kb. The *omp87* gene is represented by the pink shaded arrow. The *amp*^{R} gene is represented by the light blue shaded arrow.

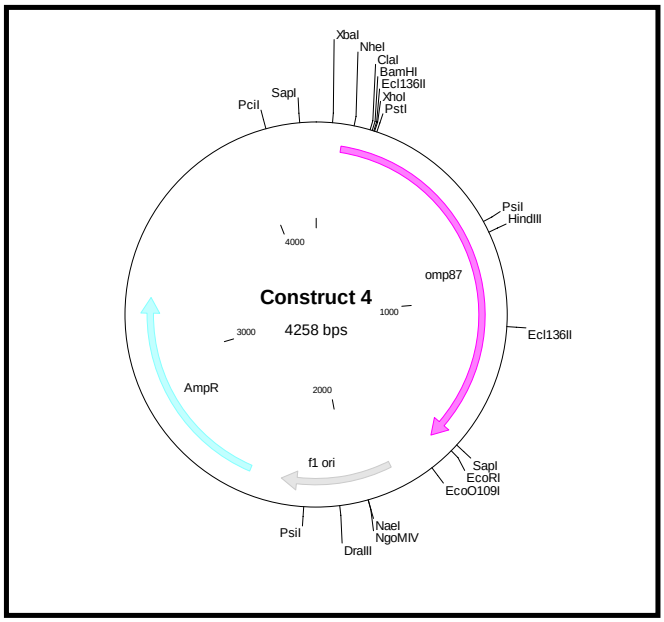


Figure 6.8 – Physical map of the plasmid pRSET, Construct 4, containing the smallest *omp87* insert of 1.5 kb. The *omp87* gene is represented by the pink shaded arrow. The *amp*^{R} gene is represented by the light blue shaded arrow.

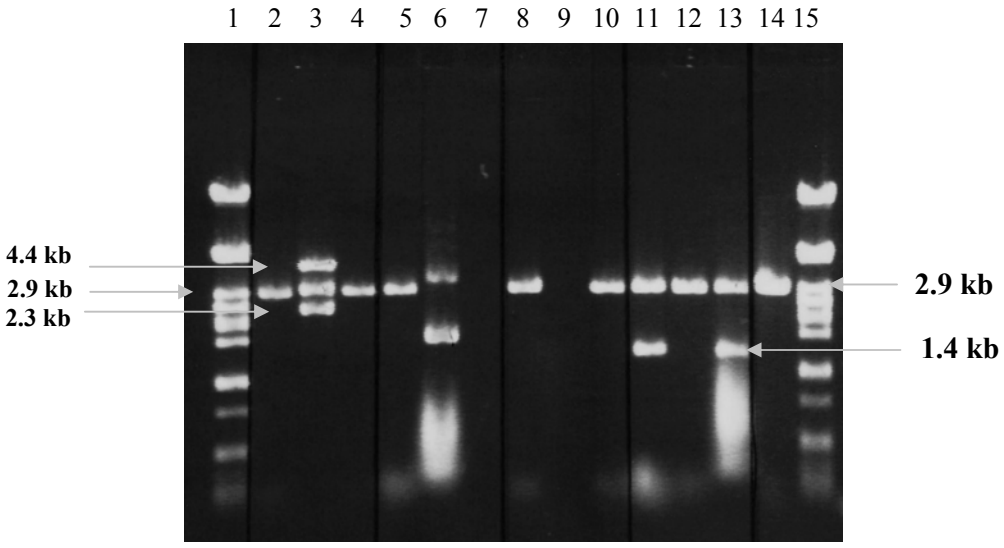


Figure 6.9 Restriction digestion of recombinant pRSETA plasmids. Lane: 1, lambda x *Pst*I marker; Lanes 2-4, pRSET fragment 1 clones x *Pst*I; Lanes 5-7, pRSET fragment 2 clones x *Pst*I; Lanes 8-10 : pRSET fragment 3 clones x *Pst*I; Lanes 11-13, pRSET fragment 4 clones x *Pst*I; Lane 14, Empty plasmid pRSET; Lane 15, lambda x *Pst*I marker.

From the above results, it was determined that one of the three clones of Construct 1 **(Figure 6.9, lane 3)** contained the expected DNA fragments, but also contained an additional, unexpected product of around 4.4 kb. This clone was therefore re-grown, the plasmid was isolated and digested. The digestion products now showed the expected sizes of 2.9 and 2.4 kb (**Figure 6.10**).

Despite several cloning attempts, Construct 2 could not be obtained. However, repeated attempts with Construct 3 did result in the desired plasmid with the expected digestion fragments (**Figure 6.11, lane 4**). Two successful clones containing a Construct 4 plasmid were obtained in the first attempt (**Figure 6.9, lanes 11 and 13**), with the expected product sizes of 2.9 kb and 1.4 kb.

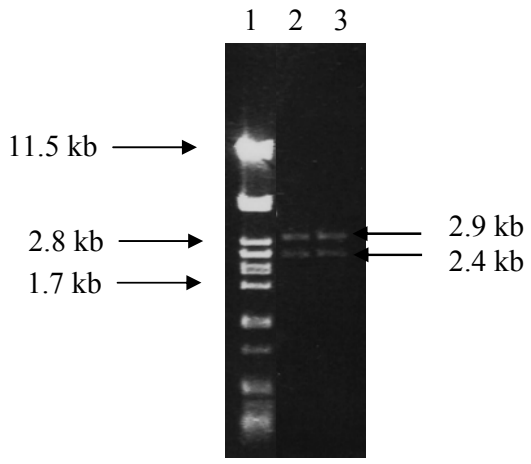


Figure 6.10. Restriction digestion of plasmid pRSETA Construct 1 clones. Lane: 1, lambda x *Pst*I marker; Lanes 2-3, Construct 1 clones digested with *Pst*I and *Eco*RI.

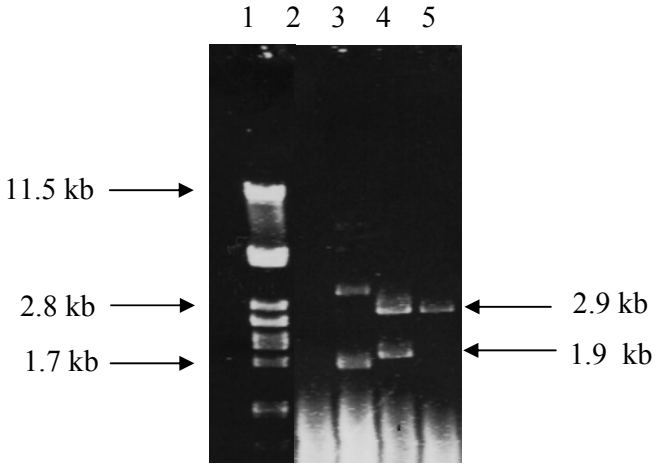


Figure 6.11. Restriction digestion of plasmid pRSETA Construct 3 clones. Lane: 1, lambda x *Pst*I marker; Lanes 2-5 Construct 3 clones digested with *Pst*I and *Eco*RI.

6.3.5 Sequencing of Omp87 recombinant protein DNA

In order to ensure that the 6 x Histidine tag was in the correct reading frame relative to the Omp87 truncates, the pRSET plasmids of all 3 constructs were sequenced. The pRSET T7 Promoter primer, and reverse primer pair were used for the DNA sequencing reactions.

The sequencing was performed successfully, and revealed that the Omp87 truncates had been successfully cloned into the correct reading frame with the 6 Histidine residues of the pRSET vector. The expressed proteins would therefore contain the 6 x His-tag that would enable them to be purified using the Ni-NTA column.

6.3.6 Growth and induction of pRSET plasmid constructs

Following the successful cloning of three truncated Omp87 constructs in pRSET, analysis of the protein products could now commence by inducing the expression of the genes in the host cell *E. coli* BL21.

As the pRSET vector contains a polylinker region **(Figure 6.3)**, cloning of the amplified PCR product truncates into this region results in a larger insert size (i.e. a fusion protein), by 120 bp. Following ligation, the expected sizes of the fusion proteins are as follows:

Construct 1: 2495 bp (831 amino acids - 92 KDa)
Construct 2: 2342 bp (780 amino acids - 86 KDa)
Construct 3: 2030 bp (676 amino acids - 75 KDa)
Construct 4: 1511 bp (503 amino acids - 55 KDa)

The induction time required for maximal protein expression is one variable which differs between protein expression systems. The recommended guidelines outlined by Invitrogen (www.invitrogen.com) for the expression of cloned proteins from the pRSET expression system were therefore optimised for maximal protein expression.

The induction of the pRSET constructs was performed at hourly intervals over 5 hours, and some samples were left overnight. The samples were then separated on a polyacrylamide gel, by SDS-PAGE. Samples containing induced Construct 1, the largest

Omp87 construct, did not show any expression of the protein, over any of the induction hours tested, including the overnight sample. The results for construct 1 are shown in **Figure 6.12**. The results for constructs 3 and 4, which did show expression of the truncated proteins are shown in **Figures 6.13** and **6.14**.

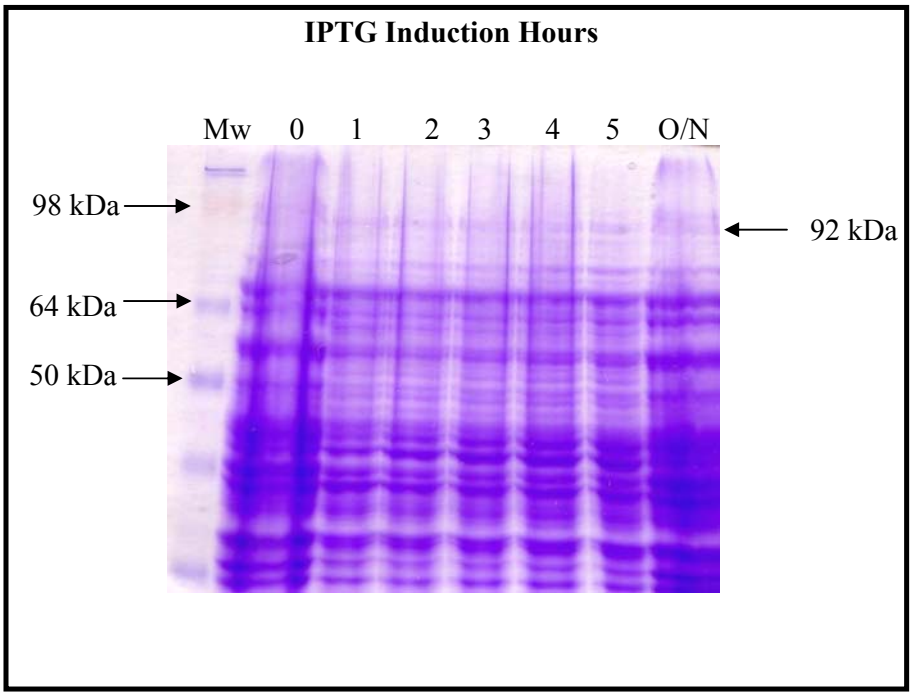


Figure 6.12. SDS-PAGE gel of induction trial with Omp87 construct 1. No additional expression of a 92 kDa protein can be seen over the time frame tested. Lane Mw: SeeBlue® protein molecular marker; Lane 0: Induction time = 0 hrs; Lane 1: Induction time = 1 hr; Lane 2: Induction time = 2 hrs; Lane 3: Induction time = 3 hrs; Lane 4: Induction time = 4 hrs; Lane 5: Induction time = 5 hrs; Lane O/N : Induction time = overnight.

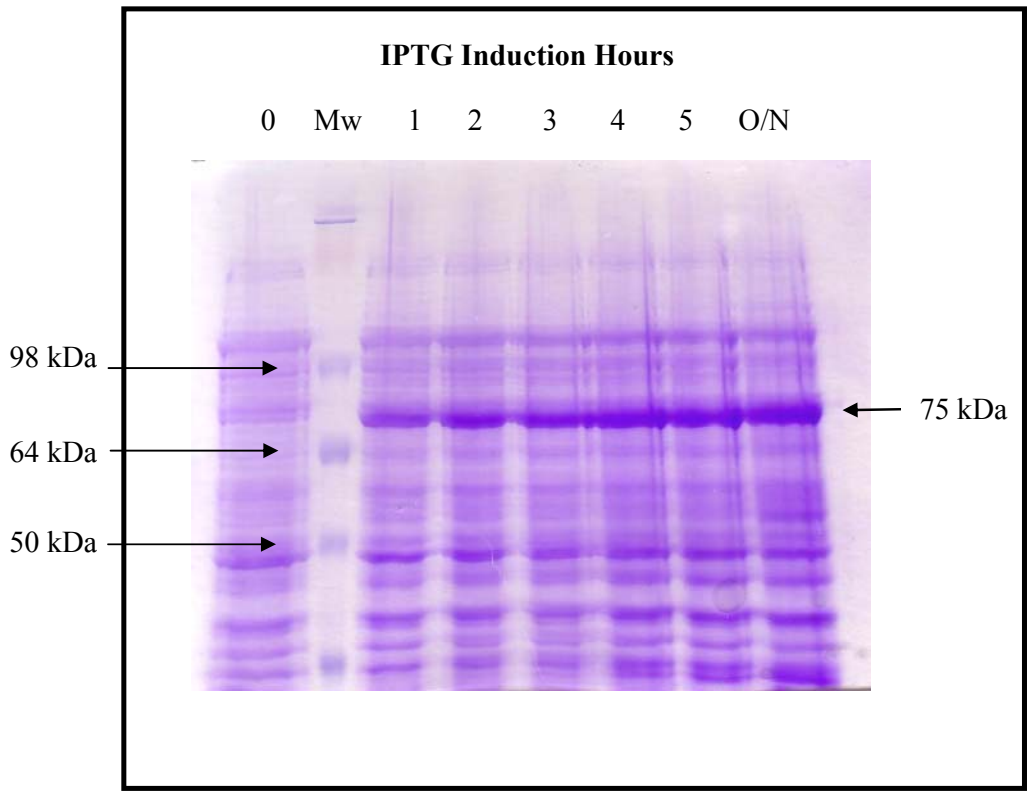


Figure 6.13. SDS-PAGE gel of induction trial with Omp87 construct 3. The concentration of the expressed 75 kDa Omp87 truncate can be seen to increase over the time frame tested. Lane 0: Induction time = 0 hrs; Lane Mw: SeeBlue® protein molecular marker; Lane 1: Induction time = 1 hr; Lane 2: Induction time = 2 hrs; Lane 3: Induction time = 3 hrs; Lane 4: Induction time = 4 hrs; Lane 5: Induction time = 5 hrs; Lane O/N: Induction time = overnight.

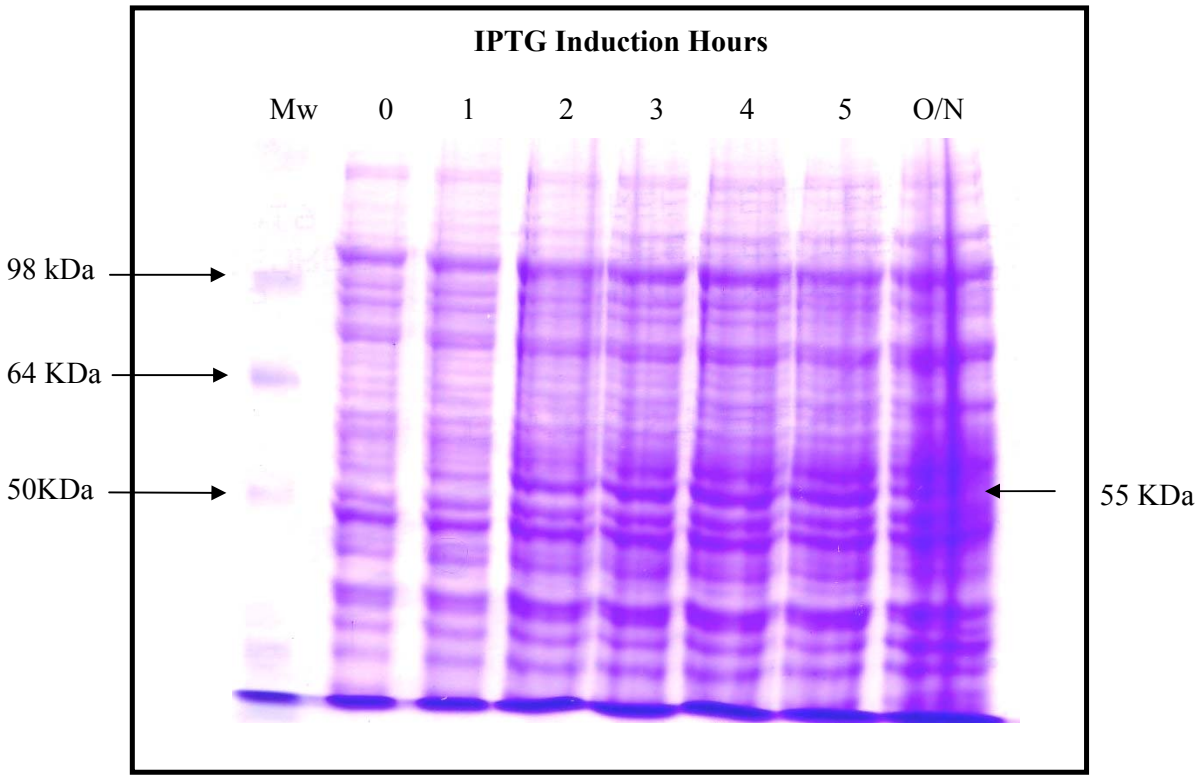


Figure 6.14. SDS-PAGE gel of induction trial with Omp87 construct 4. The concentration of the expressed 55 kDa Omp87 truncate can be seen to increase over the time frame tested. Lane Mw: SeeBlue® protein molecular marker; Lane 0: Induction time = 0 hrs; Lane 1: Induction time = 1 hr; Lane 2: Induction time = 2 hrs; Lane 3: Induction time = 3 hrs; Lane 4: Induction time = 4 hrs; Lane 5: Induction time = 5 hrs; Lane O/N : Induction time = overnight.

The results with the expression of constructs 3 and 4 show that the concentration of expressed protein did not increase after 4 hours of induction with IPTG. An induction time of 4 hours was therefore selected for the remainder of the study, as the optimal induction time.

For the different IPTG concentrations investigated, there was very little difference observed between the concentrations of 1 mM, 5 mM and 10 mM (results not shown). Below 1 mM, the expression of the protein was slightly lower. It was therefore decided that 1 mM was the optimal IPTG concentration for use with the recombinant pRSET constructs.

The results of the optimal induction temperature were also analysed. There was a slight difference observed with the yield of protein obtained from the 28°C and 32°C induction temperatures (results not shown). However, this difference was not only limited to the expressed recombinant protein. It was instead an overall reduction in total cell protein amounts, due to the slower growth rate of cells at these reduced temperatures. When this reduction in recombinant protein expression was viewed relative to the overall reduction in total cell protein, the difference in recombinant expression was negligible. Therefore, although the cells grew at a slower rate at the lower temperatures, the expression of the recombinant protein was virtually uniform over the temperature ranges tested.

6.3.6.1 Determination of truncated Omp87 protein solubility

In order to determine whether the expressed Omp87 proteins were soluble or insoluble, the *E. coli* cells containing the pRSET constructs were induced for 4 hours with 1 mM IPTG. The cells were then boiled in SDS-PAGE loading buffer containing β-mercaptoethanol. The samples were then centrifuged, and both the supernatant and pellet were analysed by SDS-PAGE.

β-mercaptoethanol is a reducing agent which reduces the disulfide bonds within proteins and usually leads to their complete unfolding and linearisation. If a protein is insoluble, however, the protein aggregates into a dense, biologically inactive form that requires a very high concentration of denaturant or detergent to dissolve the protein into an unfolded, linear structure.

The results indicated that the truncated proteins were insoluble, as they were present in the pelleted fraction of the sample (results not shown).

The School of Chemical Engineering and Materials Science at the University of Oklahoma (http://biotech.ou.edu/) have devised a program which determines the likelihood of the solubility of a recombinant protein when over-expressed in *E. coli*. The protein sequences were entered into the program, and the predicted percentages of the protein solubility or insolubility was determined. This program was used for an estimate of the solubility of all pRSET Omp87 constructs.

The results from the Recombinant protein solubility prediction program are shown in **Table 6.4**.

Table 6.4. Results for each Omp87 construct from the Recombinant protein solubility prediction program (http://biotech.ou.edu/). The protein sequence of the recombinant protein is entered into the program, and the program then determines the predicted solubility or insolubility for that particular protein when overexpressed in *E. coli*. The CV-CV' value denotes the likelihood that the recombinant protein is soluble in the cytoplasm of *E. coli* (CV-CV' < 0) or will form inclusion bodies (CV-CV' > 0) (Wilkinson and Harrison, 1991).

Construct	Protein Size (aa)	CV-CV' value	Predicted % Solubility/Insolubility
Construct 1	831 aa	2.43	93.3 % Insoluble
Construct 3	676 aa	2.26	91.7 % Insoluble
Construct 4	503 aa	2.29	92 % Insoluble

6.3.7 Western blotting of Omp87 recombinant protein using anti-His antibody

Immunoblotting was performed with antibody which specifically binds to histidine tags. Anti-His antibody was used to determine if the His-tag was being correctly expressed. The results revealed reactivity with the proteins. However there was also reactivity with several other smaller proteins in the samples, particularly in the pre-column sample. This could indicate that there was binding of the anti-his antibody to intrinsic histidine residues in other proteins or peptides; or it may have indicated that there was proteolytic breakdown of the recombinant proteins and the anti-his antibody was recognizing, and binding to these smaller fragments.

In order to address the potential problem of protein degradation, the protease inhibiting enzymes Leupeptin and Benzamidine were added to the pre-column preparation steps, and were used at a final concentration of 1 μg/ml and 1 nM, respectively. However no significant difference in reactivity was observed.

6.3.8 Purification of truncated Omp87 proteins by gravity-flow chromatography

A sepharose Ni-NTA column purification method was used for the purification of the recombinant Omp87 proteins constructs. Purification of the expressed proteins through this purification system proved to require a substantial amount of optimisation in order to obtain the final eluted protein in a relatively pure state, with a reasonably high yield. An initial concentration of 6 M Urea was used for the solubilisation of the protein, but this was not sufficient, as the protein was not effectively binding to the column. The higher concentration of 8 M Urea was therefore used, and proved to be effective. Also, an initial concentration of 250 mM imidazole was used for eluting the proteins from the column. This was not successful, and the protein remained bound to the column following the final elution steps. This concentration was therefore increased to 500 mM imidazole, and again, proved to be successful (results not shown).

SDS-PAGE results for the purification of both Omp87 constructs 3 and 4 are shown in **Figures 6.15 and 6.16**.

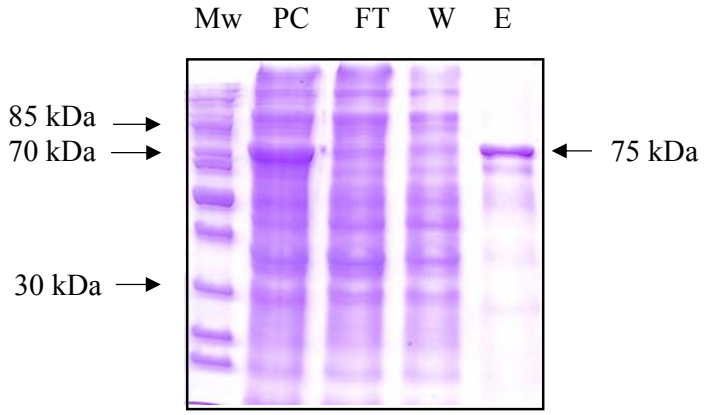


Figure 6.15. SDS-PAGE analysis of protein fractions from the purification of Omp87 construct 3, using a Ni-NTA column. Lane Mw, molecular weight standard, Lane PC, Pre-column fraction; Lane FT, Flow-through fraction; Lane W, wash fraction; Lane E, Eluted fraction.

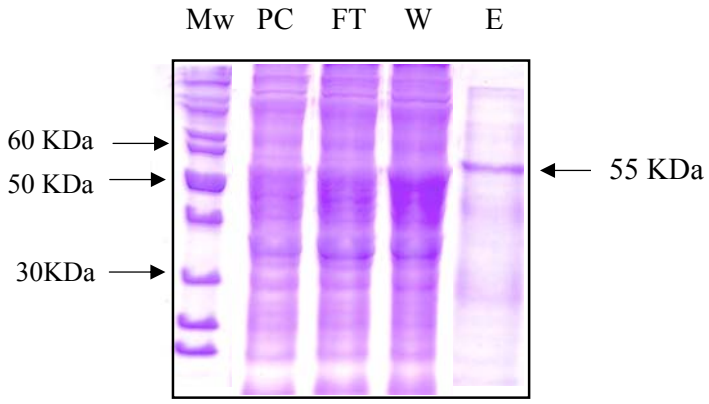


Figure 6.16. SDS-PAGE analysis of protein fractions from the purification of Omp87 construct 4, using a Ni-NTA column. Lane Mw, molecular weight standard, Lane PC, Pre-column fraction; Lane FT, Flow-through fraction; Lane W, wash fraction; Lane E, Eluted fraction.

Through the introduction of several more washing steps to the column, a purer yield of the final eluted protein product was able to be obtained. The SDS-PAGE analysis of the final purification products of Omp87 constructs 3 and 4 are shown in **Figure 6.17.**

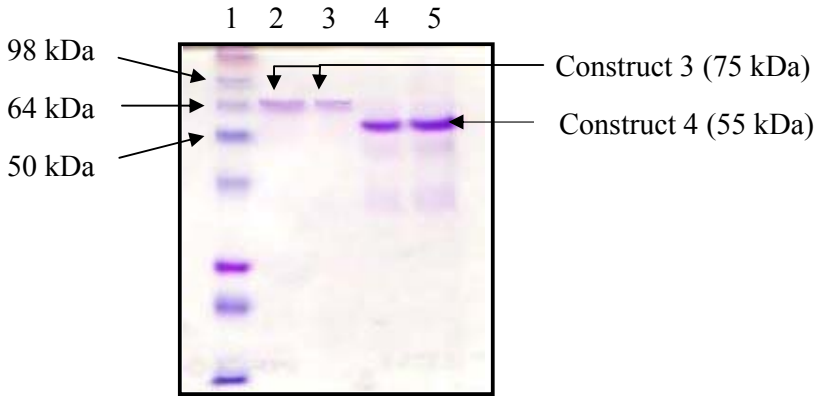


Figure 6.17. SDS-PAGE analysis of eluted protein fractions for Omp87 constructs 3 and 4, using a Ni-NTA column. Lane 1, SeeBlue® protein molecular marker; Lanes 2-3, Eluted fractions from construct 3; Lanes 4-5, Eluted fractions from construct 4.

6.3.9 Immunoblotting of recombinant Omp87 constructs

In order to determine if the expressed recombinant proteins were recognised by human anti-*L. pneumophila* antiserum, immunoblotting was performed.

A whole cell lysate of *L. pneumophila* AA100 was also included as a control sample. The results of the immunoblot are shown in **Figure 6.18.**

As expected, the *L. pneumophila* whole cell lysate control sample shows clear reactivity with the antiserum. Constructs 3 and 4 also show reactivity with the human antiserum, indicating that the proteins are still recognised by the antiserum, despite being truncated versions of the original protein, with an incorporated histidine tag.

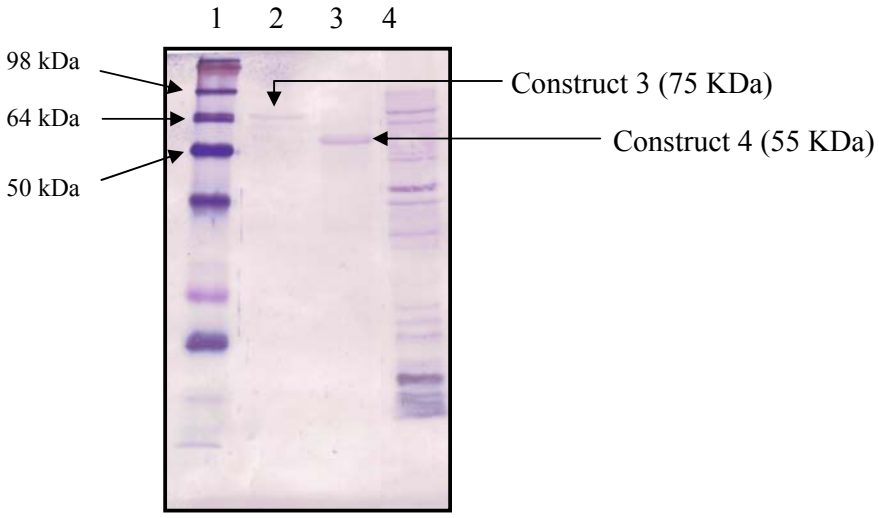


Figure 6.18. Immunoblot of recombinant pRSET protein constructs 3 and 4 with human anti-*L. pneumophila* antiserum. Lane 1, SeeBlue® protein molecular marker; Lane 2, recombinant construct 3 protein; Lane 3, recombinant construct 4 protein; Lane 4, *L. pneumophila* AA100 whole cell lysate.

6.4 Discussion

In order to express the *omp87* gene of *L. pneumophila*, four different fragments of the gene were cloned into the expression vector pRSET. Cloning of the pRSET Omp87 was successful for the smaller protein fragments 3 and 4. However, this was not the case with the two larger constructs 1 and 2. Omp87 construct 1 was obtained following the co-isolation of this plasmid in combination with a plasmid containing an unknown insert of 4.4 kb.

Despite many attempts, no successful clones were obtained containing the construct 2 insert. This may have been due to the fact that larger fragment sizes in plasmids are usually more difficult to clone, or it may have been due to the fact that these larger clones also encoded for the protein's secretory signal sequence. This may have meant that the exporting of these proteins to the outer membrane of the *E. coli* cell following protein translation destabilised the organism with lethal consequences. An accumulation of proteins on the cells outer membrane may have been an excessive structural load on the cell, thereby rendering these clones unstable. Although we would not expect this to occur in the absence of the inducer IPTG, there is often still a small amount of 'leaky' expression occurring. Although pRSET construct 2 was truncated, it was only around five amino acids smaller than the entire Omp87 protein. As we do not know the exact location of the protein's secretory signal, it may indeed be the case that PRSET construct 2 also encodes for the secretory signal of the protein. Through sequence analysis, we have only been able to predict that the secretory signal of the Omp87 protein is located at the N-terminal region of the protein, and is around 44 amino acids in length (Chapter 4).

The reason we could recover a construct 1 clone was perhaps due to mutations or frame shifts, as no expression was observed in the induction experiments.

Mitchison *et al* (2000) also experienced difficulty cloning the entire *oma87* gene of *Pasteurella multocida*, using the plasmid pGEX-4T-3. Despite several attempts at cloning the entire gene, and the use of several different cloning vectors including pGEX and pRSET, the group resorted to cloning shorter fragments of the gene.

A histidine tag was incorporated in the reverse primers that were used to amplify fragments 2, 3 and 4. This was done to ensure that the histidine tag would be present at

the C-terminal region, in the event that post-translational modifications to the proteins would result in cleavage at the N-terminal region of the protein, and hence cleavage of the N-terminal histidine tag. If the histidine tag was cleaved from the translated protein, there would be no means for purification of the recombinant protein. The cleavage site of the protein is usually situated at the N-terminal region of the protein. It is at this site that proteins are usually cleaved following processing or translocation. Therefore, inclusion of the histidine tag at the C-terminal region ensured that the proteins would still be able to be purified on the Ni-NTA column, even in the event of such posttranslational modifications.

The process of protein purification was performed successfully and resulted in the isolation and purification of the recombinant proteins from pRSET Omp87 truncates 3 and 4. Amounts of protein purified varied, but were usually in the range of 3-5 mg per 200 ml of *E. coli* broth culture. These proteins had molecular weights of 75 kDa and 55 kDa respectively, and were purified to a relatively pure state through the use of the Ni-NTA chromatography system. The 75 kDa construct 3 protein was purified to a higher degree than the smaller construct 4 protein, which had a few faint bands present on the SDS-PAGE gel, indicative of proteins which had co-purified with the recombinant construct 4 protein. These additional bands could also signify protein degradation however, as the presence of the histidine tag on the breakdown products would still bind to the Ni-NTA column, and result in their purification. As both of the constructs were purified under the same conditions however (i.e. temperature, buffer composition) we would probably expect evidence of protein degradation in both constructs, and not only one.

The optimisation for the Ni-NTA column purification process was quite lengthy, particularly due to the formation of inclusion bodies, by the recombinant proteins. The formation of inclusion bodies is reportedly a common occurrence, particularly from the high level expression of recombinant proteins in host cells such as *E. coli*. Inclusion bodies can be found in both the cytoplasmic and periplasmic space of bacteria, and are described as being dense, globular and amorphous protein aggregates (Baneyx, 1999). They are believed to be one of the major impediments in protein production, and hinder the development of many areas of proteomics, such as the design and synthesis of novel proteins and the modification of natural proteins (Ventura, 2005). Inclusion bodies are

also seen however, as an efficient initial step in the purification process, as greater than 90% of the inclusion body usually consists of the recombinant protein (Clark, 2001).

Predicting whether or not a recombinant protein is likely to be insoluble, and liable to form inclusion bodies is quite a useful and valuable tool, as it may prevent the need for the initial laboratory trials of purifying the recombinant protein under both native and denaturing conditions. The program developed by The School of Chemical Engineering and Materials Science at the University of Oklahoma (http://biotech.ou.edu/) is a program devised to predict the solubility of recombinant proteins when over-expressed in *E. coli*. The proteins sequence is entered into the program, and the predicted percentage of the proteins solubility or insolubility is determined. The program is based on a statistical analysis of protein parameters such as charge average, turn forming residue fraction, cysteine fraction, proline fraction, hydrophilicity, and total number of residues, and is based on 81 proteins that either are, or are not involved in the formation of inclusion bodies. This analysis enables the program to predict the probability that a recombinant protein will form inclusion bodies based only on the amino acid sequence of the protein (Wilkinson and Harrison, 1991). The pRSET construct protein sequences showed high values of predicted insolubility. In the instance of constructs 3 and 4, this was indeed the case, as a very high concentration of urea was required to solubilise these proteins. For the construct 1 protein, a comparison cannot be made as the protein was not successfully expressed using the pRSET expression system.

During the optimisation of the protein purification process using the Ni-NTA column, a few important changes were made that had a significant impact on the success of the purification. Firstly, as both proteins were insoluble, a very high concentration of urea would be required in order to solubilise the protein. A concentration of 6 M urea was used for solubilisation during the initial stages, but the protein appeared to not bind to the column, and was instead found in the flow-through fraction.

At this stage, it had already been ascertained that the histidine tag was present, and was effectively expressed. The lack of the histidine tag could therefore be excluded as a potential cause for the apparent binding deficiency. The conformation of the protein was then considered, as the histidine tag was present, but possibly not able to be accessed, due to the conformation of the protein. If the protein had still been present in the form of a globular inclusion body, the histidine residues may have been shielded, and structurally inhibited, from binding to the Ni^{2+} residues on the column. The concentration of urea used for the solubilisation of the proteins was therefore increased

to 8M. The samples were also given additional time to bind to the column, by recapping the column following the addition of the sample. The column was then left shaking gently at room temperature for 1 hour. This gentle shaking also increased the binding potential of the sample to the Ni^{2+} residues, as it allowed for better mixing of the sample with the sepharose/ Ni^{2+}. Following this extra binding time, the sample was then allowed to pass through the column. The flow-through fraction was then collected, and again passed through the column, to ensure that maximum binding of the recombinant protein occurred.

Another alteration that was made was the increase in imidazole concentration used for elution of the protein from the column. Imidazole binds competitively with the histidine tag to the Ni^{2+} residues in the sepharose. Following these changes, the proteins were effectively binding to the column, but were not being eluted during the final elution step using 250 mM imidazole. This concentration was therefore increased to 500 mM imidazole. Elution of the protein then occurred as expected, and resulted in a relatively clean and pure product.

However, as there continued to be slight co-purification of other proteins with the recombinant Omp87 truncates, additional washing of the column was incorporated into the process, in the form of increased volume of washing buffers that were passed through the column. These washing buffers were prepared with the consecutively lower concentrations of urea that were passed progressively through the column, and appeared to reduce the presence of the co-purified proteins. The concentration of urea was reduced gradually in these washing buffers to ensure that the protein did not resolubilise during the purification process.

The SDS-PAGE analysis of the recombinant proteins gave a clear and concise indication of the success of the expression, and protein purification processes. The construct 3 protein band, of 75 kDa, was very prominent and easy to visualise on the gel. The construct 4 protein band, of 55 kDa, was not as prominent on the gel, but was still easy to distinguish from amongst the other protein bands.

Immunoblotting performed on the recombinant proteins with the human anti-*Legionella* antiserum demonstrated that the *Legionella* antibodies were still able to recognise the *Legionella* Omp87 truncated proteins, although they were truncated versions of the Omp87 protein, and contained a histidine tag. This indicated that the portion of the

protein that was included in the truncates contained immunogenic regions. It also indicated that the incorporation of the histidine tags did not interfere with the antigen-antibody binding reaction, or the conformation of the proteins.

Further work will be performed on the recombinant Omp87 proteins, including the raising of polyclonal antiserum in rabbits. This work will be described in the following chapter (Chapter 7).

CHAPTER VII

Raising antibodies to

Legionella pneumophila Omp 87:

Animal Experimentation

and

Antibody Analysis

7. Introduction

Cross-reactivity between proteins of different bacterial species is often a major factor involved in the development of diagnostic tests. Occasionally the cross-reactivity may prove helpful if the detection of the organism itself is the critical parameter, and not the species of the bacteria. However, identifying the bacterial species involved is often important, and this diagnosis may be essential in decisions made involving the treatment regime, such as the commencement of antimicrobial therapy. Often there is substantial difference between the virulence levels of different strains of bacteria. In the case of *Legionella* for instance, numerous species are highly virulent, whilst others pose no known threat to human health (Salyers and Whitt, 2002).

The Omp87 protein of *L. pneumophila* may potentially be a target for the development of diagnostic tests for this organism, or for use in a surveillance-type system to prevent outbreaks of disease in problematic sites such as cooling towers.

Raising antibodies to the Omp87 protein, and determining whether these antibodies are able to detect *L. pneumophila*, and the extent to which these antibodies cross-react with other species of *Legionella*, is therefore necessary as a preliminary investigation into the value of this protein as a target antigen for *Legionella* detection.

7.1 Outline of this chapter

This chapter describes the animal experimentation involved in raising polyclonal antibodies against the Omp87 protein of *L. pneumophila*, using the purified protein truncates produced in Chapter 6. Four New Zealand White rabbits were used for producing antisera, which was performed over a 12 week period. Analysis of antibody titre levels, and cross-reactivity studies were then performed using Enzyme Linked Immunosorbent Assays (ELISA). Cross-reactivity was also determined using SDS-PAGE and Immunoblotting, using different *Legionella* species and *L. pneumophila* serogroups.

7.2 Materials and Methods

7.2.1 Preparation of Omp87 protein samples for raising of polyclonal antiserum

Following the purification of the protein truncates (Chapter 6), the protein concentration was increased by centrifuging the samples at 3,000 x *g* for 1 hour in a YM-30 Centricon concentrating device, (Amicon, Inc., U.S.A) with a 30 kDa cut-off limit. Any remaining urea and imidazole was also removed from the sample by consecutive washing of the sample with phosphate buffered saline (PBS) in the centricon device. Precipitated protein was removed from the filter of the device and kept, so as to allow the continuous flow through of the sample.

The concentration of the precipitated protein sample removed from the centricon was then determined by performing a Lowry assay (see Chapter 2, Materials and Methods). The protein amount was then adjusted to 200 μg using PBS, in a total volume of less than 1 ml per rabbit.

7.2.2 Animal experiments

Four 9-week-old female New Zealand White rabbits were used for raising the polyclonal antisera. The procedure utilised was approved by the University Animal Ethics Committee.

7.2.2.1 Pre-bleed blood collection and processing

Fifteen milliliters of blood was taken prior to the commencement of the study by catheterisation of the central ear artery. The blood was collected and left overnight at 4°C to clot. The following day, the tubes were centrifuged at 5,000 x *g* for 15 minutes. The supernatant was then collected and the pellet was discarded. The serum was again centrifuged at 5,000 x *g* for 15 minutes to remove any remaining red blood cells. The serum was then aliquoted into 1 ml aliquots and stored at -20°C.

7.2.2.2 Administration of antigen injections and bleeds

The first injection was given as a 1:1 ratio of antigen/PBS:Freunds Complete Adjuvant (FCA). The follow up booster administrations were given at the same ratio, except that Freunds Incomplete Adjuvant (FIA) was used, instead of FCA. The mixing of the protein/PBS and CFA was done by using 2 x 2 ml glass syringes connected via a 3- way tap. The mixture was mixed with the syringes for 25 minutes. For the follow up booster injections, the protein/PBS and FIA was emulsified by sonication. The mixture was sonicated on ice at 35% amplitude for a total of 45 seconds. With both methods, the consistency of the mixture was checked by placing a drop of the mixture into a petri dish containing H_2O. The emulsification was deemed sufficient if the drop was able to retain its shape in the liquid.

The injection inoculum was divided into 3 equal parts and the injections were administered at different sites subcutaneously along the flanking sides of the rabbits.

The first injection was given at week 1, followed by booster injections at weeks 3, 5, 7, and 9. Bleeds were taken by catheterisation of the central ear artery at weeks 0 (pre-bleed), 8, 11 and 13. Following the final bleed at week 13, all rabbits were euthanised.

7.2.3 Immunoblotting for analysis of pre-bleed serum

Immunoblotting was used to analyse the pre-bleed sera. Samples separated using SDS-PAGE included 30 μg of whole cell lysate samples of *E. coli* and *L. pneumophila*, and 1 μg of purified Construct 3 and Construct 4 proteins. The SDS-PAGE gel was then used for immunoblotting with the pre-bleed antiserum. The pre-bleed rabbit serum was diluted to 1/12 with Tris-buffered Saline/Tween20 (0.05%) (TST). A secondary antibody was used at a 1/1000 dilution (in TST) of goat, anti-rabbit IgG, conjugated with Horse-radish peroxidase (HRP). The bound peroxidase was then visualised by developing the blot with 4-chloro-1-napthol in tris-buffered saline (TBS).

7.2.4 Absorption of antiserum with *E. coli* whole cells and whole cell lysates

For the absorption of antiserum with whole *E. coli* cells, a fresh 10 ml broth culture of *E. coli* BL21 cells was centrifuged at 5000 x *g* for 15 min. The pellet was washed 2 x with PBS. The pellet was then collected and incubated with 10 ml of rabbit antiserum, which had been diluted 1/10 with PBS. The mixture was then left shaking gently overnight at 4°C. For the absorption of antiserum with whole cell lysates, 10 ml of fresh broth culture of *E. coli* BL21 cells was centrifuged at 5000 x *g* for 15 min. The pellet was washed 2 x with PBS. The pellet was then resuspended in 5 ml of PBS. The suspension was then sonicated at maximum amplitude for 6 cycles x 15 seconds, on ice. Five hundred microliters of the cell lysate was then added to 10 ml of rabbit antiserum which had been diluted 1:10 with PBS. With both methods, following the overnight absorption of the antiserum, the antiserum was centrifuged at 5,000 x *g* for 10 minutes. It was then filtered using a 0.45 μm filter, followed by filtration with a 0.2 μm filter (Gelman Sciences, U.S.A).

7.2.5 ELISA assays for determination of antiserum titre

Indirect ELISA assays were performed to determine the titre of antibodies in the post-immune sera. Firstly, 3 μg of each recombinant antigen was prepared in ELISA coating buffer (0.15 M Na_2CO_3, 0.35 $NaHCO_3$, pH 9.6). This was added to wells of a 96-well plate and left incubating at room temperature for 2 hours, or overnight at 4°C. Plates were then rinsed 5 x with Phosphate buffered saline/Tween20 (PBST). The ELISA plate was then blocked using 1% BSA in PBS. This was incubated at room temperature for 30 minutes, or overnight at 4°C. The plates were then rinsed 5 x with PBST. Doubling dilutions of antiserum were then added to wells (diluted in PBS/0.05% Tween-20). Dilutions ranged from 1/100 to 1/204,800. This was incubated for 2 hours at room temperature, or 1 hour at 37°C. The secondary antibody used was a 1/1000 dilution (in TST) of HRP conjugated goat, anti-rabbit IgG. This was then added to wells, and was left for 2 hours at room temperature, or 1 hour at 37°C. The plates were again rinsed 5 x with PBST. The plates were then developed by adding the substrate solution 3,3',5,5'-tetramethylbenzidine (TMB) (BD Biosciences, U.S.A). The plates were allowed to

develop for up to 1 hour. The reaction was then stopped by the addition of 2M H_2SO_4. The plates were then immediately read at 450 nm using a microplate spectrophotometer. Control samples were also included in the ELISA. These were: 'no antigen added to well ', 'no blocking of well', and 'no conjugate added to well'.

7.2.6 Western blotting for the determination of cross-reactivity

Immunoblotting was used for the determination of antiserum cross-reactivity. Organisms used for the cross-reactivity testing consisted of different *Legionella* serogroups and species, and different bacterial species. These organisms are shown in **Table 7.1.** Whole cell lysates of all organisms were prepared, (as described in Chapter 2, Materials and Methods) and the concentration of each protein sample was determined by performing a Lowry assay, as described by Markwell *et al* (1978). Thirty micrograms of cell lysate was then separated by SDS-PAGE, and this gel was used for immunoblotting with the antiserum of rabbit 2. A dilution of 1/500 (in TST) of the primary antiserum (rabbit) was used. The secondary antiserum used was a 1/1000 dilution (in TST) of goat, anti-rabbit IgG, conjugated with Horse-radish peroxidase (HRP). The bound peroxidase was then visualised by developing the blot with 4-chloro-1-napthol in tris-buffered saline (TBS).

Table 7.1 Bacterial serogroups and species included in cross-reactivity studies

Organism	Source
L. pneumophila serogroup 1-13	University of Kentucky*
L. gratiana	"
L. cincinnatiensis	"
L. wadsworthii	"
L. adelaidensis	"
L. micdadei rivera	"
L. santicrucis	"
L. longbeacheae	"
L. birminghamensis	"
L. dumoffii	"
L. cherrii	"
L. spiritensis	"
L. parisensis	"
L. maceachernii	"
Shigella flexneri	RMIT University**
Salmonella typhimurium	"
Haemophilus influenzae	"
Aeromonas hydrophila	"
Pasteurella multocida	"
Moraxella catarrhalis	"
Vibrio parahaemolyticus	"
Pseudomonas aeruginosa	"
Serratia marcesens	"
Xanthomonas campestris	"

* Strains kindly provided by Prof. Yousef Abu Kwaik, University of Kentucky, U.S.A

** Strains kindly provided by Ms Celia M^{c}Kenzie, RMIT University, Australia

7.2.7 Fluorescence Microscopy

Fluorescence microscopy was performed in order to visualise antigen-antibody binding. The rabbit Omp87 antiserum was used as the primary antiserum (diluted 1:10 in PBS), and a FITC-labelled goat, anti-rabbit IgG was used as the secondary antibody (diluted 1:10 in PBS). A control primary antiserum (diluted 1:100 in PBS) was also used, which was a rabbit polyclonal antiserum against *L. pneumophila* whole cells (kindly provided by E. Grixti, School of Applied Sciences, RMIT University, Melbourne, Australia).

Samples used for the fluorescence microscopy were whole cells of *Staphylococcus epidermidis* and *L. pneumophila* (resuspended in PBS), purified recombinant protein construct 3, and over-expressed *E. coli* harbouring the pRSET plasmid either with or without the recombinant construct 3 protein insert. Controls were also used which consisted of bacterial samples with only FITC-labelled secondary antibody and PBS. This was to ensure that secondary antibody could not non-specifically bind to the bacterial cells. These images were not included in the results as no evidence of fluorescence was seen in the samples. All samples included in the fluorescence microscopy are shown in **Table 7.2**. Half a loop full of fresh bacterial culture, and 1 µg of purified recombinant protein were used. Prior to their use, the recombinant constructs were over-expressed with 1mM IPTG for 4 hours to ensure that there was maximal protein expression.

The bacterial cells and recombinant protein were attached to glass slides by fixation in 2% paraformaldehyde, pH 7.0. The slides were left to air dry for 1 hour at RT. The primary antiserum was then added, and slides were incubated at 37°C for 1 hour in a humid chamber. The slides were then rinsed by gentle agitation for 5 seconds in a petri dish containing freshly added PBS. The secondary antibody was then added, and the slides were incubated in the dark at 37°C for 1 hour in a humid chamber. The slides were rinsed again by gentle agitation for 5 seconds in a petri dish containing freshly added PBS. The slides were then air dried in a dark chamber at RT for 1 hour. One drop of mounting medium (50% glycerol in H_2O) was then added, and the slides were viewed under a fluorescent microscope.

Table 7.2. Samples included in the fluorescence microscopy study.

Sample	**Antiserum**
S. epidermidis	None
	anti-Omp87
	FITC-labelled secondary antibody only*
L. pneumophila	None
	whole cell *L. pneumophila*
	anti-Omp87
	FITC-labelled secondary antibody only*
Purified construct 3 recombinant protein	anti-Omp87
E. coli (pPRSET + Construct 3)	None
	whole cell *L. pneumophila*
	anti-Omp87
E. coli (empty pRSET)	anti-Omp87
E. coli	FITC-labelled secondary antibody*

* These results are not shown

7.3 Results

7.3.1 Preparation of Omp87 protein samples for raising of polyclonal antiserum

In order to obtain sufficient amounts of the Omp87 protein for the production of polyclonal antiserum in rabbits, concentrating of the purified recombinant protein was required. This was performed using a YM-3 Centricon concentrating device. The device has a 30 kDa cut-off limit, so that proteins greater than 30 kDa are retained by the device, and any proteins or other reagents pass through the filter, and can be discarded. Urea and imidazole, which were used during the purification process (Chapter 6) were removed from the samples by consecutively washing the device with PBS. Due to the removal of the urea, however, the Omp87 proteins began to precipitate out of solution, and could be seen in the device as white, flaky material, situated around the filter. In order to allow the washing and concentrating process to continue, and to prevent blocking of the filter, the precipitate was removed. This (precipitated) protein sample was then used for the inoculation of the rabbits, following the determination of its concentration via a Lowry assay (Markwell *et al*, 1978).

7.3.2 Immunoblotting for analysis of pre-immune serum

Blood samples were taken from rabbits prior to the commencement of the study. These were used to determine if the rabbits contained any pre-existing antibodies to *Legionella* or *E. coli*. The pre-immune antiserum from each rabbit was tested against whole cell lysates of *L. pneumophila, E. coli*, and purified recombinant protein Construct 3 and Construct 4 in a Western blotting experiment. The SDS-PAGE gel image used for the immunoblotting, and results of the immunoblotting reaction for rabbits 1-4 are shown in **Figures 7.1** and **7.2 (a-d)** respectively.

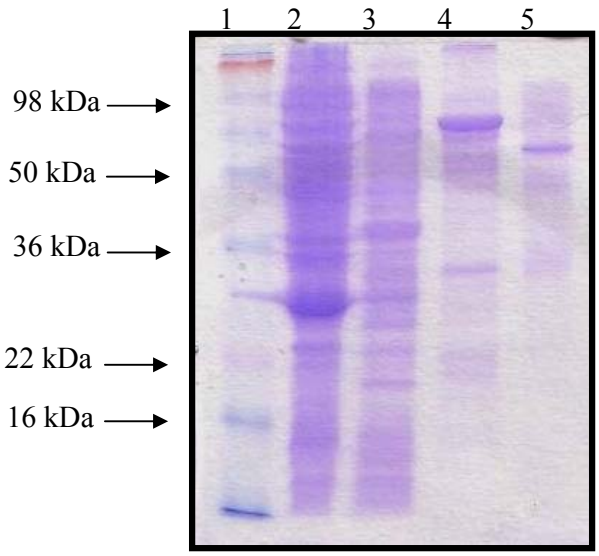


Figure 7.1. Samples separated by SDS-PAGE for immunoblotting with pre-immune antisera from rabbits 1-4. Lane 1: SeeBlue® prestained molecular weight standard (Invitrogen); Lane 2: *E. coli* BL21 whole cell lysate; Lane 3: *L. pneumophila* whole cell lysate; Lane 4: purified recombinant Construct 3; Lane 5: purified recombinant Construct 4.

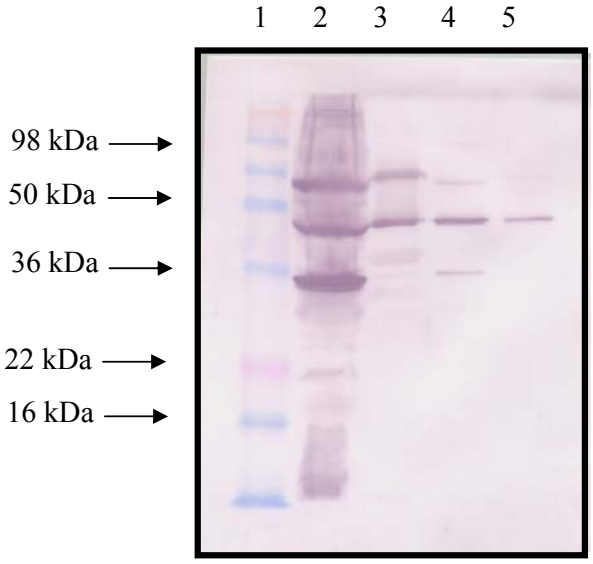


Figure 7.2 (a) Immunoblotting reaction of pre-immune antiserum from rabbit 1. Lane 1: SeeBlue® prestained molecular weight standard; Lane 2: *E. coli* BL21 whole cell lysate; Lane 3: *L. pneumophila* whole cell lysate; Lane 4: purified recombinant Construct 3; Lane 5: purified recombinant Construct 4.

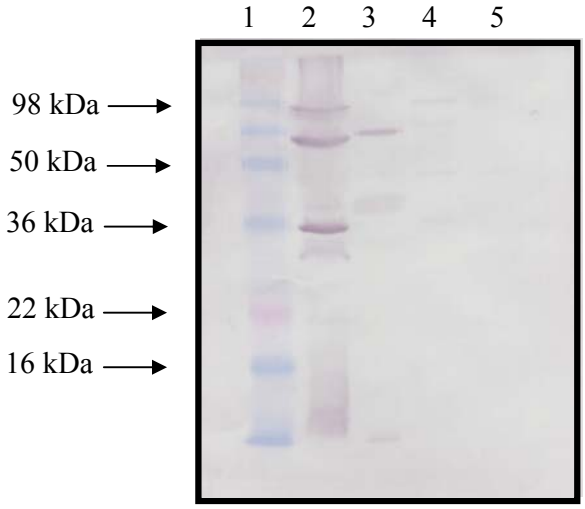


Figure 7.2 (b) Immunoblotting reaction of pre-immune antiserum from rabbit 2. Lane 1: SeeBlue® prestained molecular weight standard; Lane 2: *E. coli* BL21 whole cell lysate; Lane 3: *L. pneumophila* whole cell lysate; Lane 4: purified recombinant Construct 3; Lane 5: purified recombinant Construct 4.

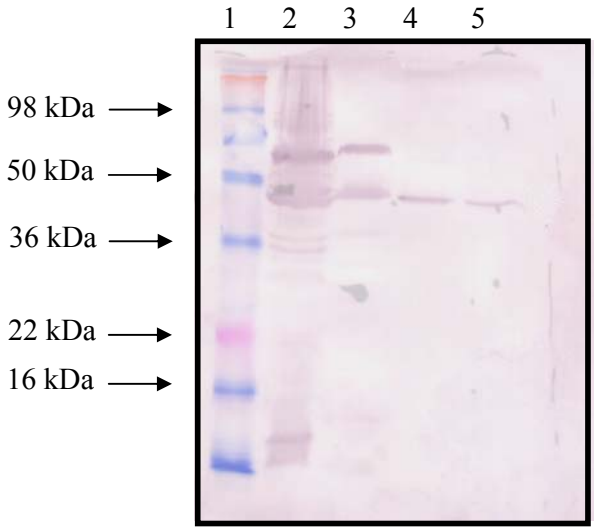


Figure 7.2 (c) Immunoblotting reaction of pre-immune antiserum from rabbit 3. Lane 1: SeeBlue® prestained molecular weight standard; Lane 2: *E. coli* BL21 whole cell lysate; Lane 3: *L. pneumophila* whole cell lysate; Lane 4: purified recombinant Construct 3; Lane 5: purified recombinant Construct 4.

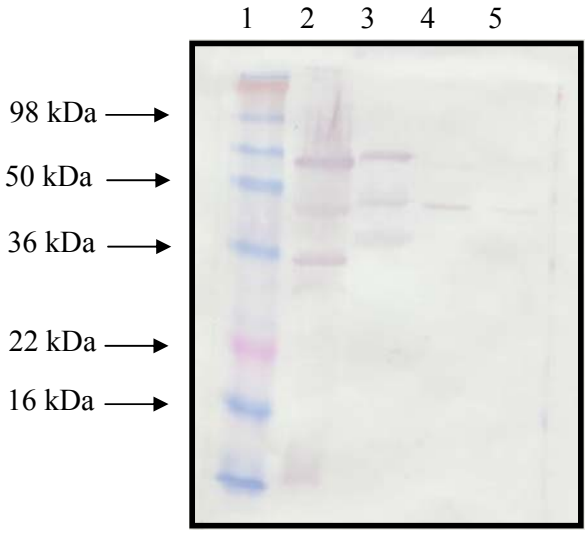


Figure 7.2 (d) Immunoblotting reaction of pre-immune antiserum from rabbit 4. Lane 1: SeeBlue® prestained molecular weight standard; Lane 2: *E. coli* BL21 whole cell lysate; Lane 3: *L. pneumophila* whole cell lysate; Lane 4: purified recombinant Construct 3; Lane 5: purified recombinant Construct 4.

The immunoblotting results show that there was reactivity with several proteins in the *E. coli* whole cell lysates (lane 2), a few proteins from the *L. pneumophila* whole cell lysate, and some of the purified constructs.

From these results, it appears that the rabbits possessed pre-existing antibodies to *E. coli*. This result was not surprising, as *E. coli* is a very common organism found ubiquitously in the environment.

There was also reactivity however, with the *L. pneumophila* cell lysate. This reactivity was not as strong as it was with the *E. coli*, and the reactivity appeared to be confined to a few bands of the same size as proteins which also reacted strongly with the *E. coli*. Due to the similarity between *E. coli* and *L. pneumophila,* it may therefore be that these bands represented *L. pneumophila* proteins that cross-reacted with antibodies against *E. coli* proteins.

Weak reactivity was also observed with proteins in lanes 4 and 5, from the recombinant protein samples. This can most likely be accounted for by the fact that the host cells used for the expression of the recombinant proteins were *E. coli*. These reactive proteins are therefore most likely to be *E. coli* proteins which were co-purified with the *L. pneumophila* recombinant proteins. Although these reactive proteins do not appear strongly on the Coomassie Blue stained SDS-PAGE gel used for the immunoblotting, they are most likely to be present in the sample. They are only seen more clearly in the immunoblot due to the high sensitivity of the technique compared to that of Coomassie Blue staining of proteins.

In order to remove these cross-reacting proteins, and to determine if the reactivity observed with *L. pneumophila* and the recombinant proteins was indeed a case of cross-reactivity, the pre-immune antiserum was absorbed with both *E. coli* whole cells and *E. coli* cell lysates.

7.3.3 Absorption of antiserum with *E. coli* whole cells and whole cell lysates

With the aim of removing cross-reactive *E. coli* proteins, and to determine if the cross-reactivity with *L. pneumophila* observed in 7.4.2 was due to *E. coli* antibodies, the pre-immune antiserum was incubated with *E. coli* whole cells and cell lysate. If *E. coli* antibodies were present in the antiserum, they would bind to the cells or cell

components, and could effectively be removed from the antiserum. Both whole cells and cell lysate were used for the absorption of antibodies to ensure that all *E. coli* proteins both on the cell surface and within the cell, would be exposed to the antiserum, and was available for the binding, and hence removal, of the antibodies.

Following the absorption process, the immunoblotting was performed with the same SDS-PAGE protein samples, which consisted of whole cell lysates of *L. pneumophila,* and *E.coli*, and purified recombinant protein Construct 3 and Construct 4 (**Figure 7.1**). The results for the immunoblotting with the *E. coli* BL21 absorbed pre-immune antiserum from all rabbits are shown in **Figures 7.3** (a-d).

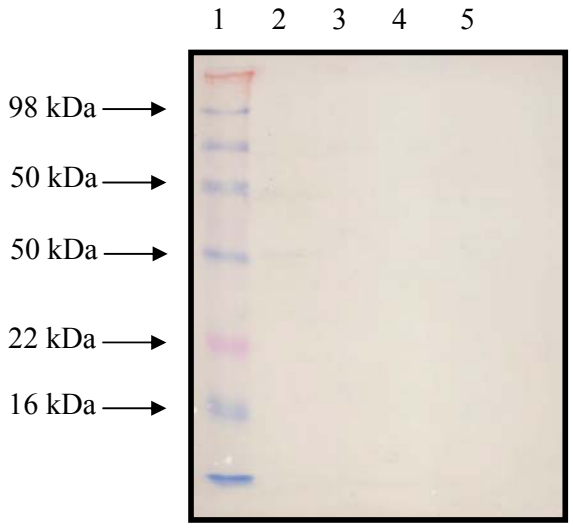


Figure 7.3 (a) Immunoblotting performed with absorbed pre-immune antiserum from rabbit 1. Lane 1: SeeBlue® prestained molecular weight standard; Lane 2: *E. coli* BL21 whole cell lysate; Lane 3: *L. pneumophila* whole cell lysate; Lane 4: purified recombinant Construct 3; Lane 5: purified recombinant Construct 4.

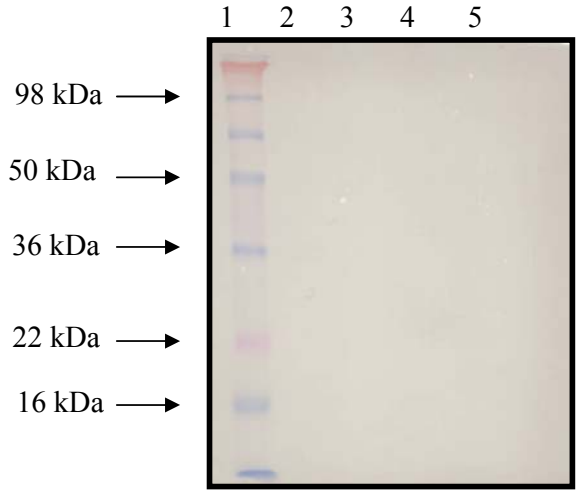


Figure 7.3 (b) Immunoblotting performed with absorbed pre-immune antiserum from rabbit 2. Lane 1: SeeBlue® prestained molecular weight standard; Lane 2: *E. coli* BL21 whole cell lysate; Lane 3: *L. pneumophila* whole cell lysate; Lane 4: Purified recombinant Construct 3; Lane 5: Purified recombinant Construct 4.

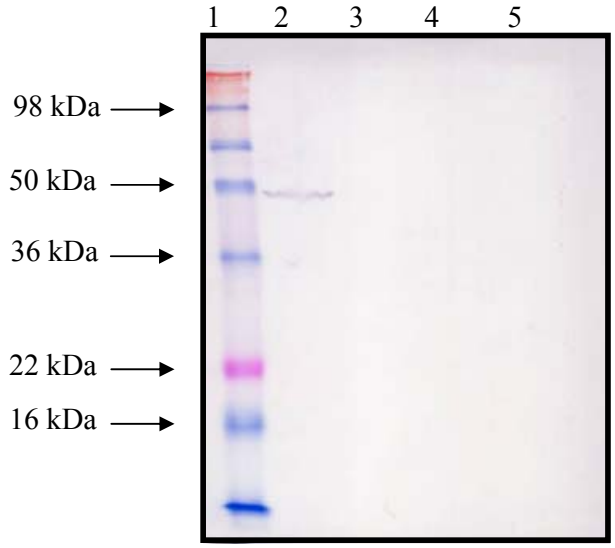


Figure 7.3 (c) Immunoblotting performed with absorbed pre-immune antiserum from rabbit 3. Lane 1: SeeBlue® prestained molecular weight standard; Lane 2: *E. coli* BL21 whole cell lysate; Lane 3: *L. pneumophila* whole cell lysate; Lane 4: Purified recombinant Construct 3; Lane 5: Purified recombinant Construct 4.

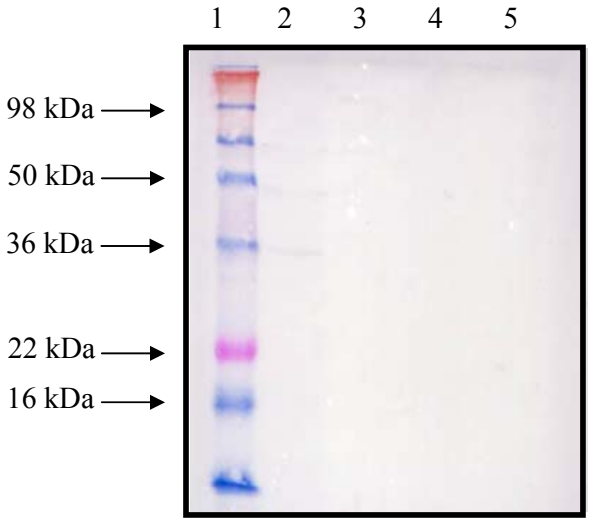


Figure 7.3 (d) Immunoblotting performed with absorbed pre-immune antiserum from rabbit 4. Lane 1: SeeBlue® prestained molecular weight standard; Lane 2: *E. coli* BL21 whole cell lysate; Lane 3: *L. pneumophila* whole cell lysate; Lane 4: Purified recombinant Construct 3; Lane 5: Purified recombinant Construct 4.

The reactivity with the pre-immune antiserum was dramatically reduced following the absorption of the antiserum with *E. coli* B21 cells and cell lysate. The reactivity dropped to little, if any, with any of the samples. This indicated that the reactivity seen with the *L. pneumophila* cell lysate in the previous immunoblots **(Figure 7.2 a-d)** was due to cross-reactivity with *E. coli* proteins, and was not due to pre-existing *L. pneumophila* antibodies in the rabbits.

7.3.4 ELISA assays for determination of antiserum titre

Four New Zealand White rabbits were used for raising polyclonal antisera against the *L. pneumophila* Omp87 recombinant proteins, Construct 3 and Construct 4. Two rabbits were used for each recombinant protein. Following the final bleed at week 13, the antisera collected were subjected to an ELISA assay to determine the titre. This was performed to enable the determination of the optimal dilution for further cross-reactivity studies using immunoblotting.

The ELISA assay required a considerable amount of optimisation, particularly in terms of antigen and antiserum concentrations used. Both higher and lower concentrations of antigen were used for coating of wells, and different ranges of antiserum dilutions were trialled. The optimal conditions for the ELISA were then determined. Dilutions of antiserum used ranged from 1/100 to 1/204,800, and 3 µg of each of the recombinant proteins was used for coating of the ELISA wells. The results obtained from the titration studies are shown in **Figures 7.4 (a-d)**. The antibody titre calculated from the assay was taken as the reciprocal of the lowest dilution demonstrating reactivity with the antigen. The conditions used for the assay are described in section 7.3.5. The background OD_{450nm} value of the assay was determined by the control sample which did not contain any antigen. This value was then subtracted from all sample results.

The data for these graphs is shown in **Appendix 5**.

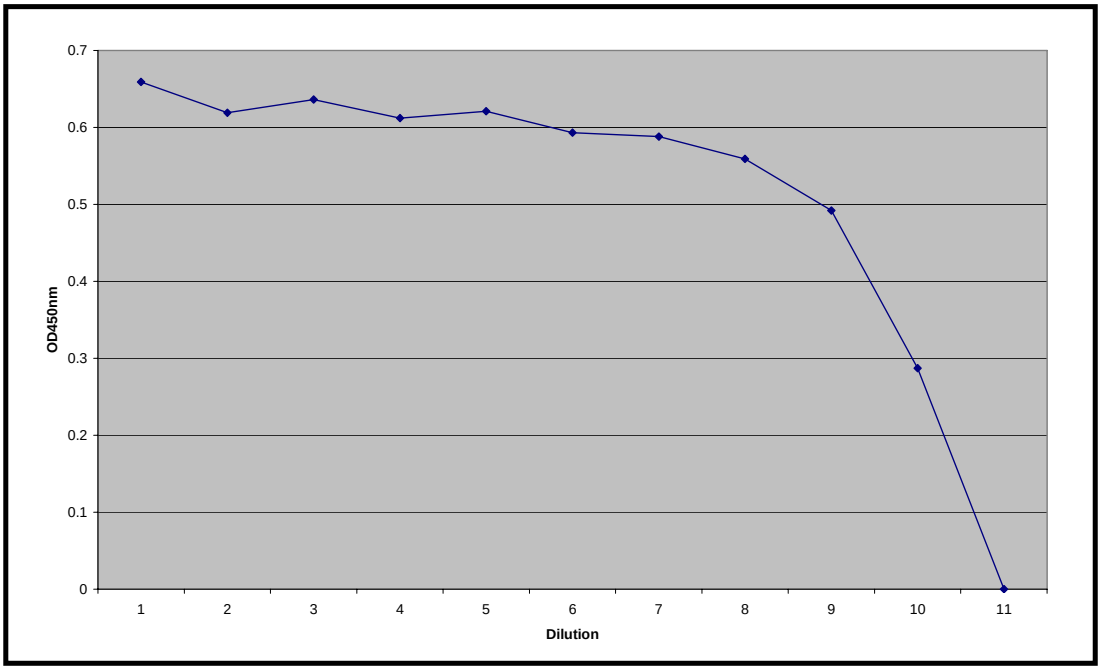


Figure 7.4 (a) ELISA titration results for terminal bleed antiserum of Rabbit 1. Dilutions were as follows: 1)1/100 2) 1/200 3) 1/400 4) 1/800 5) 1/1600 6) 1/3200 7) 1/6400 8) 1/12800 9) 1/25600 10) 1/51,200 11) 1/102400

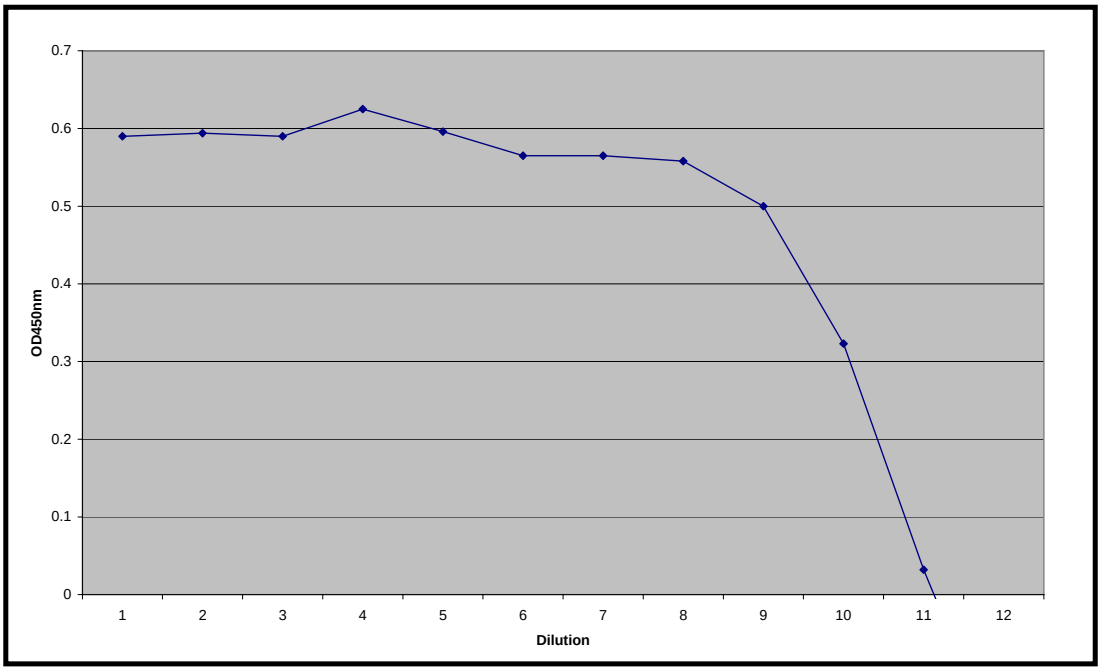


Figure 7.4 (b) ELISA titration results for terminal bleed antiserum of Rabbit 2. Dilutions were as follows: 1)1/100 2) 1/200 3) 1/400 4) 1/800 5) 1/1600 6) 1/3200 7) 1/6400 8) 1/12800 9) 1/25600 10) 1/51,200 11) 1/102400 12) 1/204800.

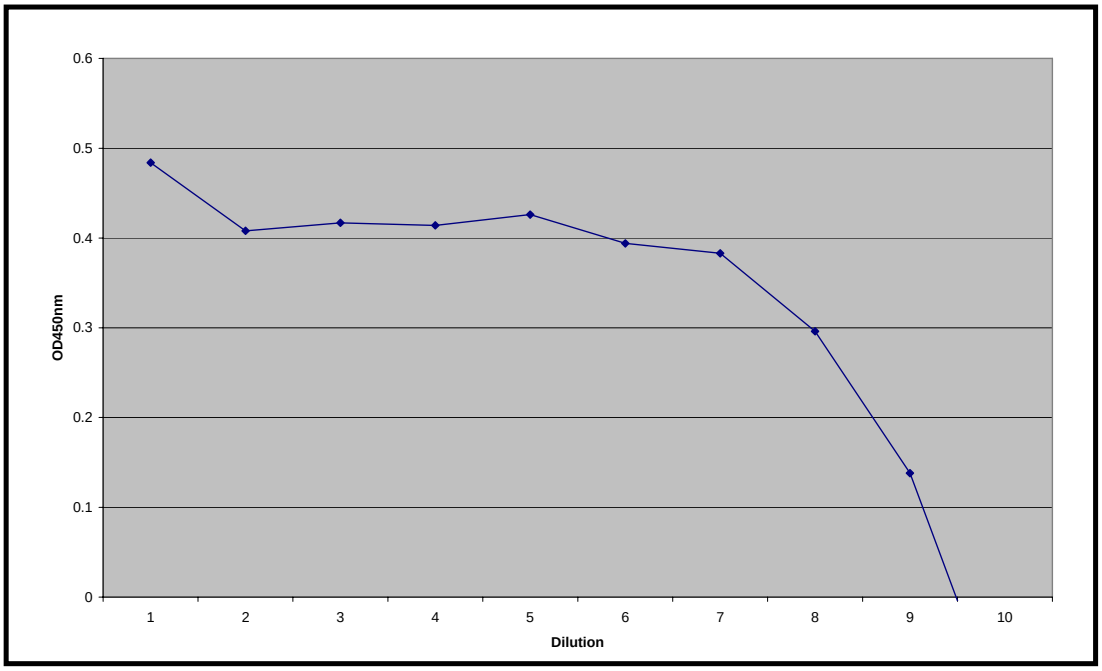


Figure 7.4 (c) ELISA titration results for terminal bleed antiserum of Rabbit 3. Dilutions were as follows: 1)1/100 2) 1/200 3) 1/400 4) 1/800 5) 1/1600 6) 1/3200 7) 1/6400 8) 1/12800 9) 1/25600 10) 1/51,200

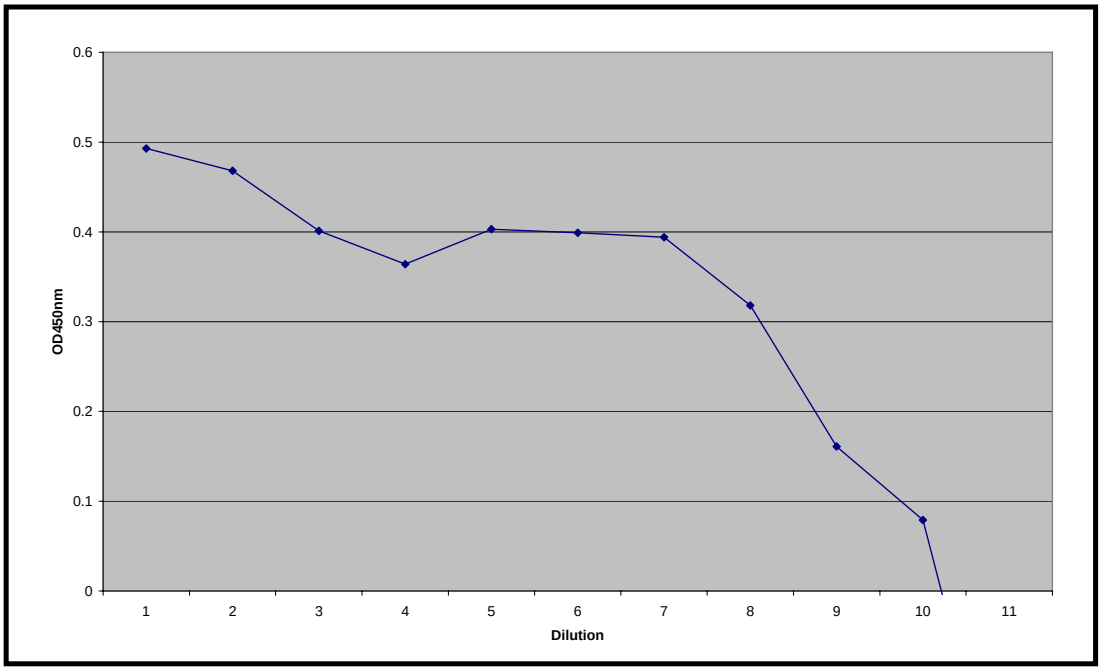


Figure 7.4 (d) ELISA titration results for terminal bleed antiserum of Rabbit 4. Dilutions were as follows: 1)1/100 2) 1/200 3) 1/400 4) 1/800 5) 1/1600 6) 1/3200 7) 1/6400 8) 1/12800 9) 1/25600 10) 1/51,200 11) 1/102400

The titre for each of the rabbit antisera was defined as the reciprocal of the last dilution to give a detectable response with the antigen. For each rabbit antisera, the titre was therefore determined to be:

Rabbit 1 Titre: 51,200

Rabbit 2 Titre: 102,400

Rabbit 3 Titre: 25,600

Rabbit 4 Titre: 51,200

7.3.5 Immunoblotting for the determination of the distribution of Omp87.

Immunoblotting was performed with the rabbit antiserum to determine if there was any cross-reactivity with different *Legionella* species and serogroups, and other related

organisms. Essentially, this study was performed to determine if cross-reactivity exists between the Omp87 protein of *L. pneumophila* and the Omp of other *Legionella* species and to determine if other closely related organisms also possess an Omp87 homologue, recognised by the *L. pneumophila* Omp87 antiserum.

Western blotting was performed with the antiserum of rabbit 2. This antiserum was selected as it was shown to possess the highest titre of antibodies (7.4.4). A dilution of 1:500 was the highest dilution used which was shown to display clear, and visible reactivity. Higher dilutions were also used, but these did not give a clear and obvious banding pattern, particularly with the more faintly reacting strains. The results of the SDS-PAGE electrophoresis and the immunoblotting for all organisms are shown in **Figure 7.5 (a-d)**.

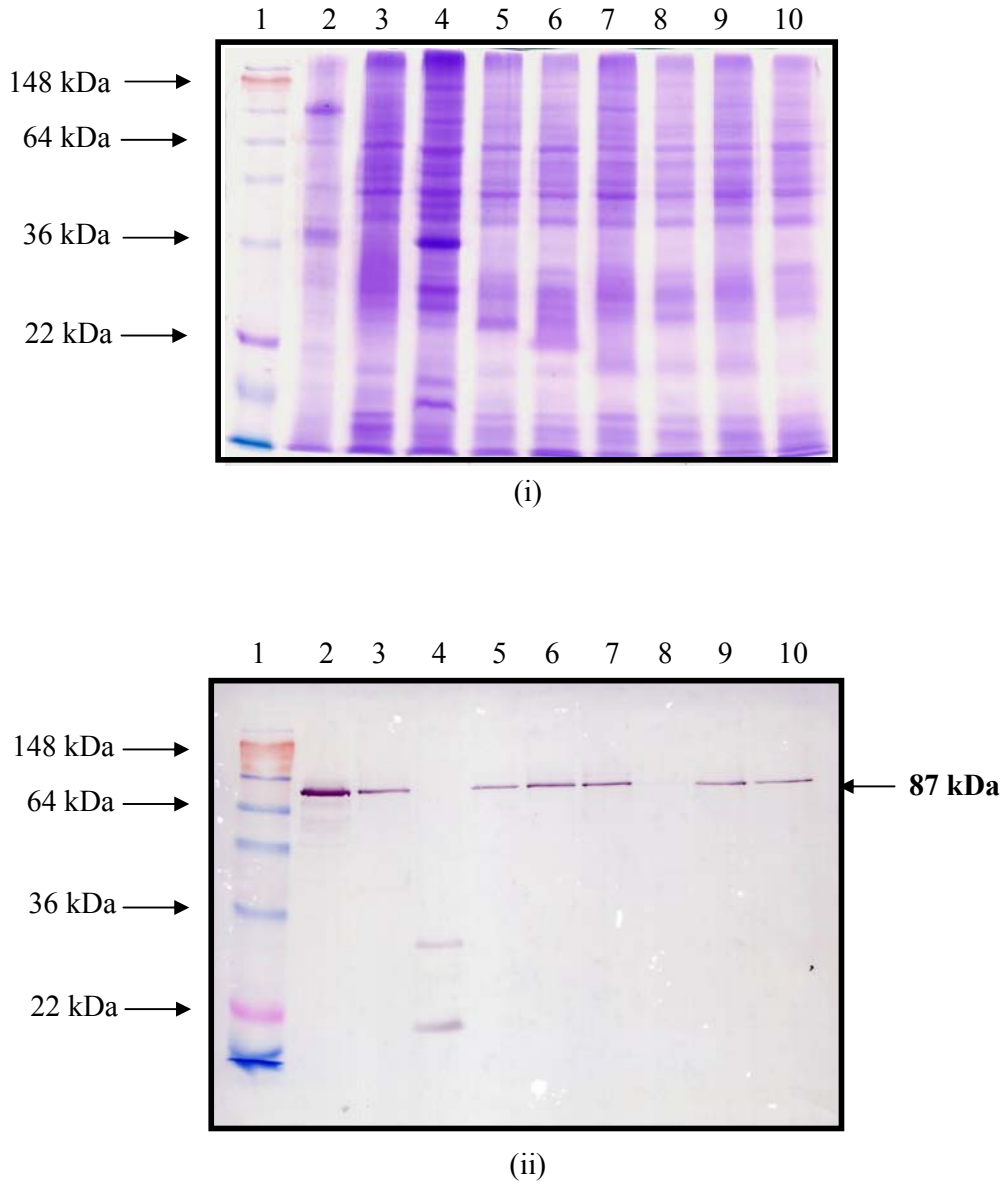
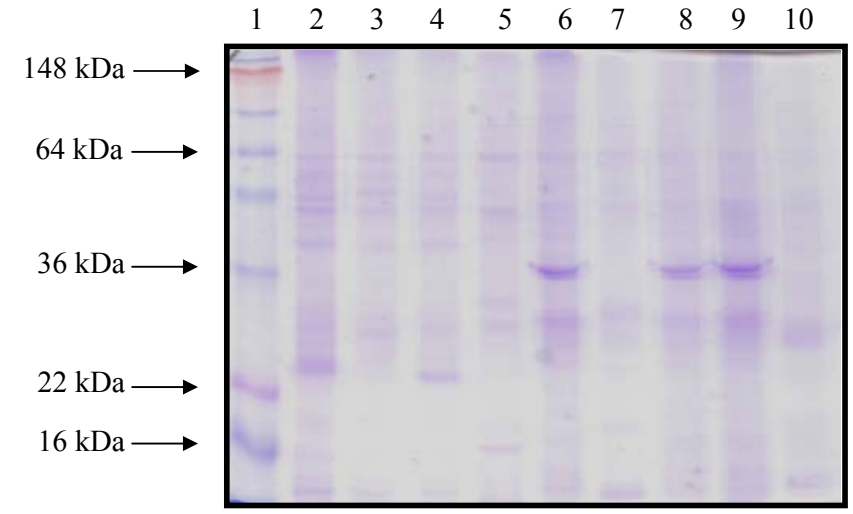
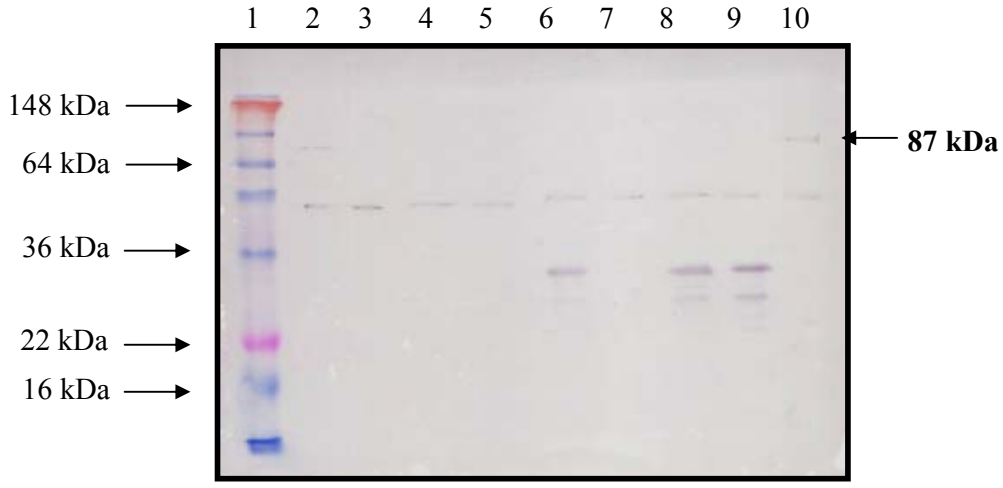


Figure 7.5 a) SDS-PAGE gel (i) and immunoblot (ii) of whole cell lysates of *L. pneumophila* serogroups 1-11. Lane 1: SeeBlue® molecular weight standard; Lane 2: *L. pneumophila* sgp 1; Lane 3: *L. pneumophila* sgp 2; Lane 4: *L. pneumophila* sgp 3; Lane 5: *L. pneumophila* sgp 5; Lane 6: *L. pneumophila* sgp 6; Lane 7: *L. pneumophila* sgp 8; Lane 8: *L. pneumophila* sgp 9; Lane 9: *L. pneumophila* sgp 10; Lane 10: *L. pneumophila* sgp 11.

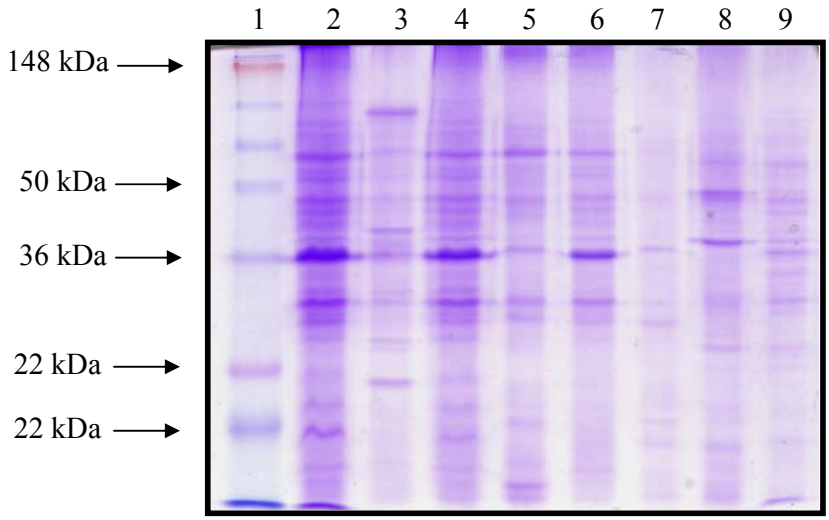


(i)

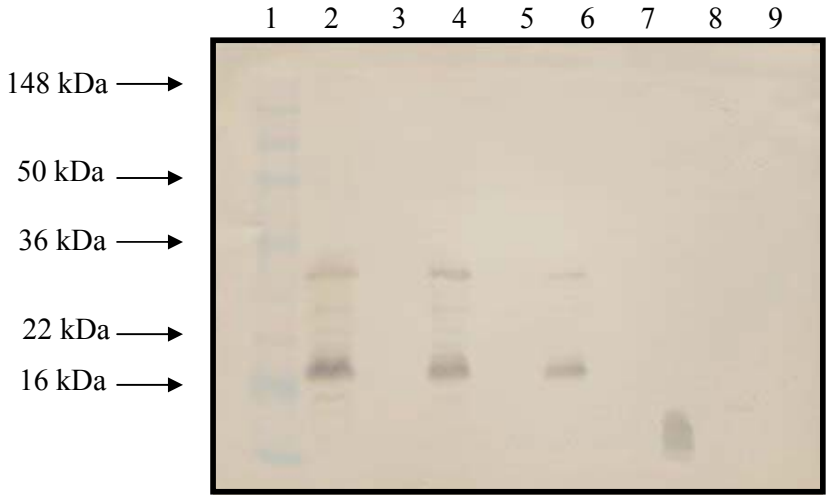


(ii)

Figure 7.5 b) SDS-PAGE gel (i) and immunoblot (ii) of whole cell lysates of *L. pneumophila* and other *Legionella* species. Lane 1: SeeBlue® molecular weight standard; Lane 2: *L. pneumophila* sgp 12; Lane 3: *L. pneumophila* sgp 13; Lane 4: *L. gratiana*; Lane 5: *L. cincinnatiensis*; Lane 6: *L. wadsworthii*; Lane 7: *L. adelaidensis*; Lane 8: *L. micdadei rivera*; Lane 9: *L. santicrucis*; Lane 10: *L. birminghamensis*.

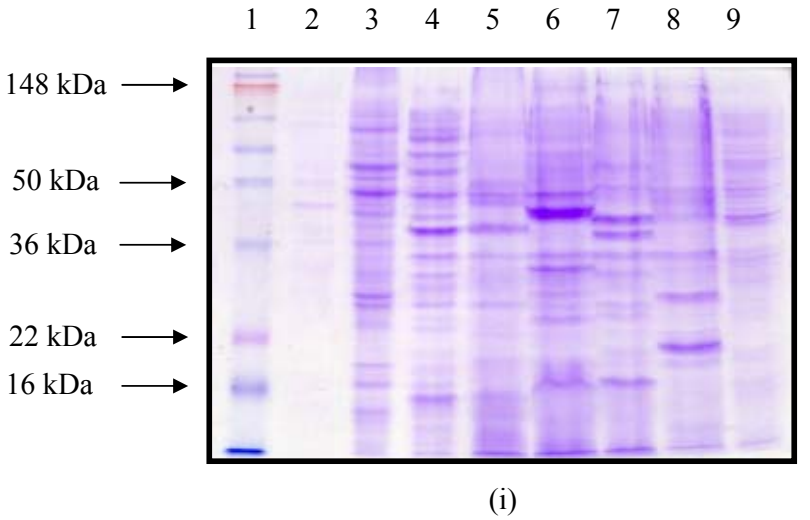


(i)

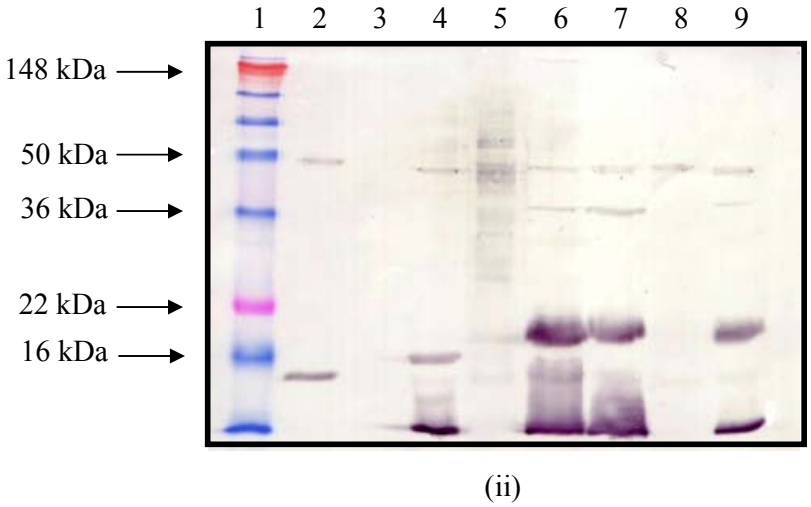


(ii)

Figure 7.5 c) SDS-PAGE gel (i) and immunoblot (ii) of whole cell lysates of different *Legionella* species. Lane 1: SeeBlue® molecular weight standard; Lane 2: *L. dumofii*; Lane 3: *L. cherrii*; Lane 4: *L. spiritensis*; Lane 5: *L. parisensis*; Lane 6: *L. maceachernii*; Lane 7: *L. longbeachae*; Lane 8: *Aeromonas hydrophila*; Lane 9: *Pasteurella multocida.*



(i)



(ii)

Figure 7.5 d) SDS-PAGE gel (i) and immunoblot (ii) of whole cell lysates of various bacterial species. Lane 1: SeeBlue® molecular weight standard; Lane 2: *Haemophilus influenzae*; Lane 3: *Moraxella catarrhalis*; Lane 4: *Vibrio parahaemolyticus*; Lane 5: *Pseudomonas aeruginosa*; Lane 6: *Serratia marcescens*; Lane 7: *Shigella flexneri*; Lane 8: *Xanthomonas campestris*; Lane 9: *Salmonella typhimurium.*

The immunoblotting showed reactivity with almost all strains of bacteria included in the cross-reactivity study. The banding pattern was different for numerous strains, particularly the non-*Legionella* organisms. Almost all of the *L. pneumophila* serogroups 1-13 displayed reactivity at the expected Omp87 size of 87 kDa, except *L. pneumophila* serogroup 3 (Lane 4) and 13 (Lane 3) which showed bands at smaller sizes, and *L. pneumophila* serogroup 9 (Lane 8) which did not show any reactivity. The smaller fragments of reactivity observed for *L. pneumophila* serogroup 3 may have been due to protein breakdown or degradation, in which case the antibodies still recognise and bind to the individual fragments of the protein, but the reactivity profile appears different to that expected. It may also be that these strains have smaller proteins with essentially the same functionality.

Reactivity was observed with most other species of *Legionella*, except for *L. cherrii, L. parisensis* and *L. longbeachae.* Reactivity at 87 kDa was only observed with *L. birminghamensis*.The banding pattern seen with these other *Legionella* strains was different, however there did appear to be some similarity with the patterns observed between these strains.

With the other bacterial species included in the study, reactivity with the antiserum was observed with *Aeromonas hydrophila, Haemophilus influenzae, Vibrio parahaemolyticus, Pseudomonas aeruginosa, Serratia marcescens, Shigella flexneri, Xanthomonas campestris,* and *Salmonella typhimurium.* The banding pattern observed with many of these organisms was similar, with a protein of around 50 kDa reacting, in addition to several smaller protein of less than 20 KDa.

7.3.6 Fluorescence Microscopy

Fluorescence microscopy was performed in order to visualise antigen-antibody binding. The observation of antibody binding with whole cells of organisms would indicate that the *L. pneumophila* Omp87 protein was surface bound, and was stearically able to bind to the antibody. The results of the fluorescence microscopy for all samples is shown in **Figures 7.6-7.15**.

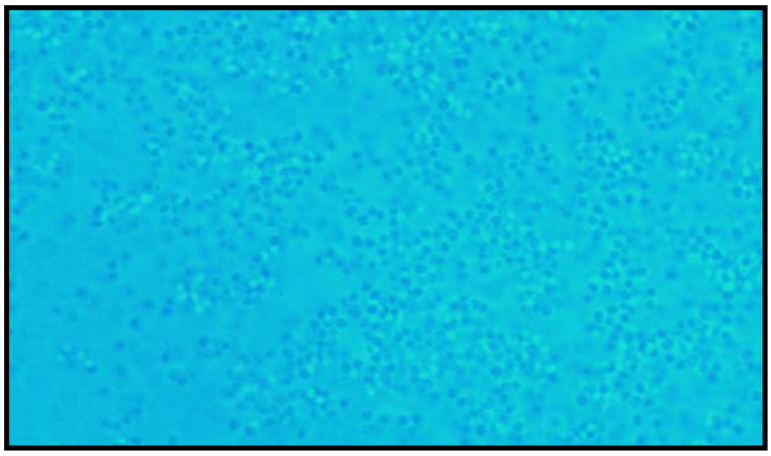


Figure 7.6. Light microscopy image of *S. epidermidis* (x 400), mounted in 50% glycerol mounting medium.

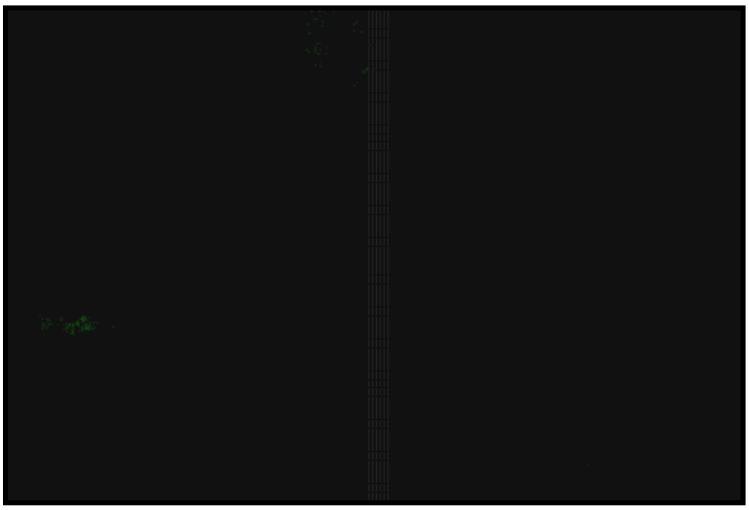


Figure 7.7. UV microscopy image of *S. epidermidis* + polyclonal anti-Omp87 recombinant protein construct 3 antiserum + FITC labelled secondary antibody (x 200).

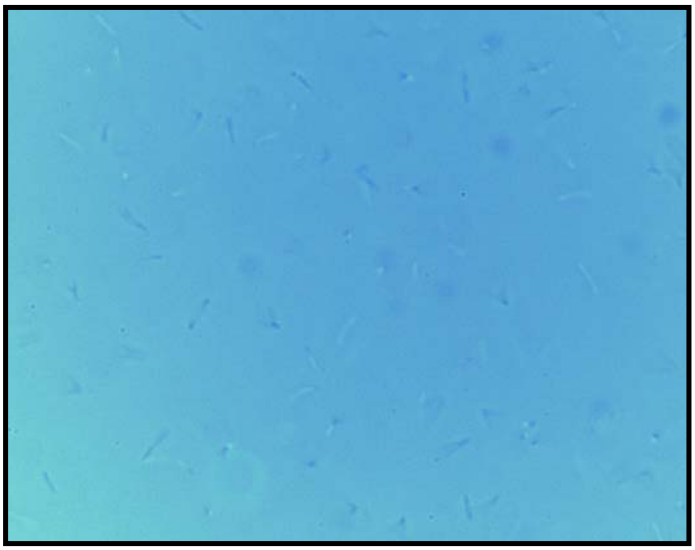


Figure 7.8. Light microscopy image of *L. pneumophila* (x 400), mounted in 50% glycerol mounting medium.

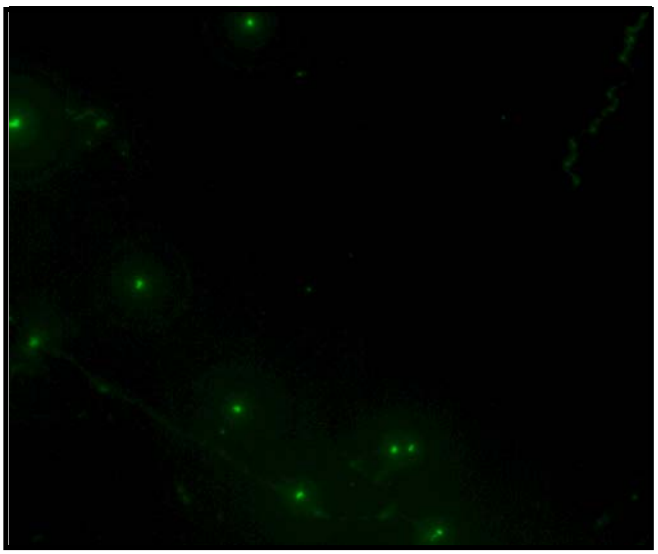


Figure 7.9. UV microscopy image of *L. pneumophila* whole cells + whole cell primary *L. pneumophila* polyclonal antiserum + FITC labelled secondary antibody (x 200).

Figure 7.10. UV microscopy image of *L. pneumophila* whole cells x polyclonal anti-Omp87 recombinant protein construct 3 antiserum + FITC-labelled secondary antibody (x 200).

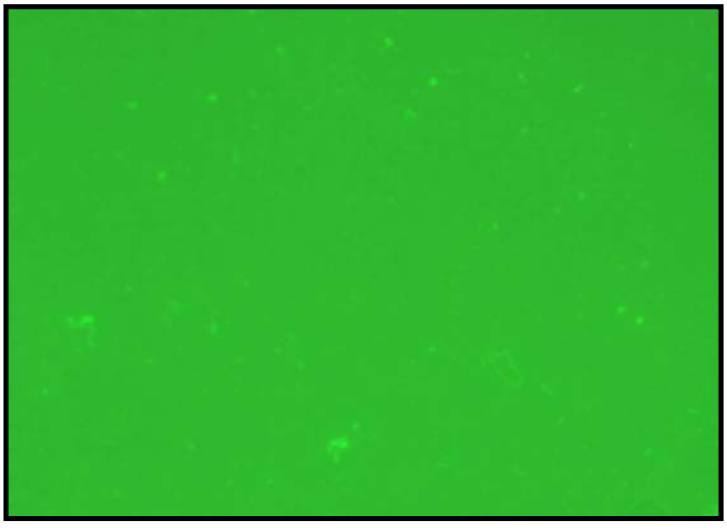


Figure 7.11. UV microscopy image of purified construct 3 x polyclonal anti-Omp87 recombinant protein construct 3 antiserum + FITC-labelled secondary antibody (x 200).

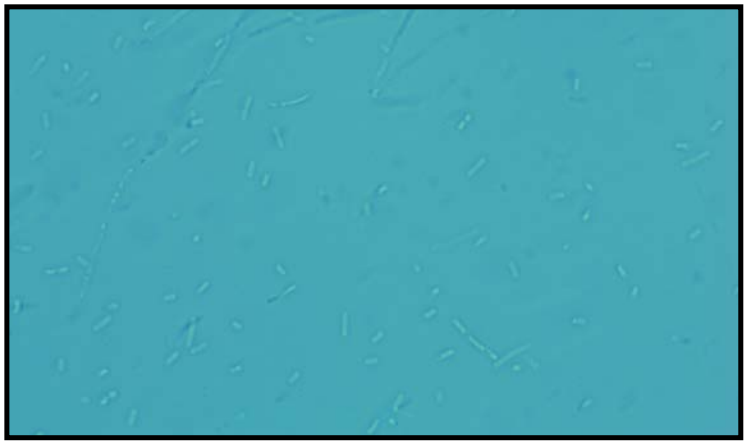


Figure 7.12. Light microscopy image of over-expressed *E. coli* harbouring construct 3 recombinant protein plasmid (x 400), mounted in 50% glycerol mounting medium.

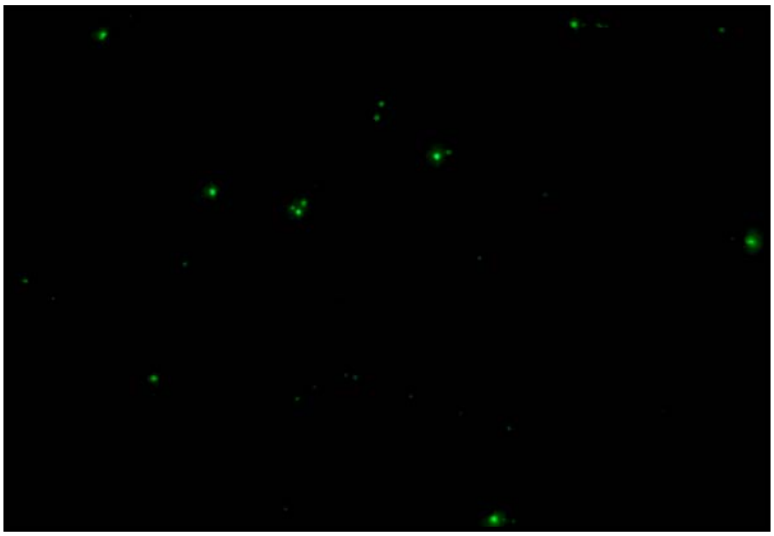


Figure 7.13. UV microscopy image of *E. co*li over-expressing recombinant protein construct 3 (in pRSET) + whole cell primary *L. pneumophila* polyclonal antiserum + FITC labelled secondary antibody (x 200).

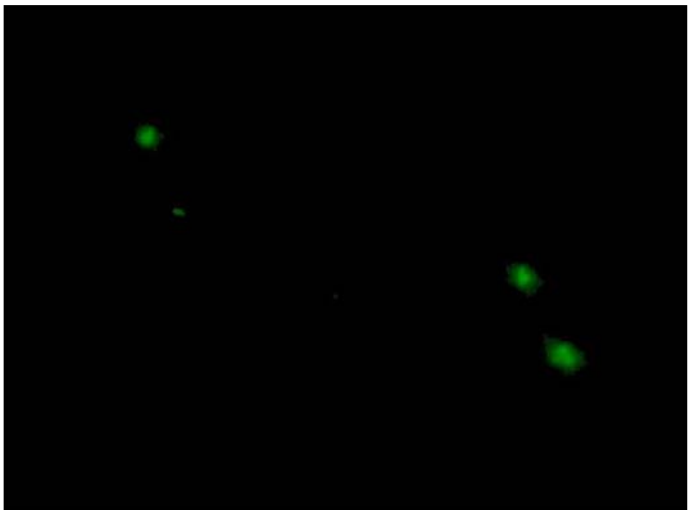


Figure 7.14. UV microscopy image of *E. co*li over-expressing recombinant protein construct 3 (in pRSET) + polyclonal anti-Omp87 recombinant protein construct 3 antiserum + FITC labelled secondary antibody (x 200).

Figure 7.15. UV microscopy image of *E. co*li harbouring empty plasmid pRSET x polyclonal anti-Omp87 recombinant protein construct 3 antiserum + FITC labelled secondary antibody (x 200).

The fluorescence microscopy images of the control samples are all as expected. The *S. epidermidis* sample did not show any reactivity with the polyclonal anti-Omp87 antiserum (Fig 7.7). Conversely, the purified construct 3 recombinant protein which was incubated with the polyclonal anti-Omp87 antiserum showed a great deal of reactivity (Fig 7.11). The entire field was highly reactive, and appeared very bright green. As anticipated, the UV microscopy image of *L. pneumophila* whole cells which were incubated with the *L. pneumophila* whole cell polyclonal antiserum also showed reactivity (Fig 7.9). The whole cell antiserum was raised against cell lysate preparations of *L. pneumophila*, so antibodies were present in this antiserum which recognised all antigenic components of *L. pneumophila*. The *L. pneumophila* cells which were incubated with the polyclonal anti-Omp87 antiserum however, did not show any reactivity (Fig 7.10). This was unexpected, as we would anticipate that the anti-Omp 87 antiserum would bind with the Omp87 protein on the surface of the *L. pneumophila* cells.

The over-expressed *E. coli* cells harbouring the recombinant protein in pRSET displayed reactivity with both the whole cell *L. pneumophila* antiserum (Fig 7.13), and the anti-Omp87 antiserum (Fig 7.14). This would indicate that the *E. coli* expressed the recombinant Omp87 protein on the cell surface, enabling it to bind to the antibodies in the antiserum. The *E. coli* cells harbouring the empty pRSET plasmid did not show any reactivity with the anti-Omp87 antiserum, as expected.

7.4 Discussion

Preparation of antigen samples for the raising of antibodies in rabbits required that samples be suspended in reagents regarded as safe for inoculation into animals, such as PBS. Therefore the chemicals used in the purification process such as urea and imidazole had to be removed through filtration with a centricon concentrating device. This device allows only compounds greater than the chosen filter size to be retained by the filter. Therefore the protein could effectively be washed with PBS. Unfortunately, the lack of the denaturant urea in the washed sample caused the protein to precipitate out of solution. This precipitation caused the filtration process to slow significantly, as the filter became blocked with white flaky protein aggregates. Upon its formation, therefore, the protein precipitate was removed from the filter and the process was resumed on the remainder of the protein solution. This problem is not uncommon when dealing with membrane proteins, as they tend to have highly hydrophobic regions, and require high concentrations of detergents to remain soluble (Baneyx, 1999).

The immunoblotting performed with the pre-bleed serum taken prior to the commencement of animal work revealed that the rabbits possessed pre-existing antibodies to *E. coli*. This is to be expected as they would be very likely to come into contact with this widespread organism through a variety of sources, such as contact with animal handlers and handled animal feed. It was unexpected however, to observe that several *L. pneumophila* proteins also reacted with this serum. However as *E. coli* and *L. pneumophila* are phylogenetically related, it was assumed that the reactivity observed with *L. pneumophila* may have been due to cross-reactivity of *E. coli* and *L. pneumophila* proteins. This was particularly evident as the *L. pneumophila* reactive proteins were of the same size as the *E. coli* reactive proteins. Removing the *E. coli* specific antibodies from the serum through absorption with *E. coli* confirmed this notion, as immunoblots performed with the absorbed antisera showed a dramatic reduction in reactivity with both the *E. coli* and the *L. pneumophila* samples. The absorbed antisera were then used for the remainder of the experiments, so as to ensure that the cross-reactivity observed between *E. coli* and *L. pneumophila* did not interfere with any other experimental results.

The determination of antibody titres by ELISA for the post-immune sera was carried out successfully, and the graphical data obtained for the assay demonstrated a clear relationship between the antigen and the concentration of diluted antiserum. The titres of the antibody levels ranged from 25,600 for rabbit 3, up to 102,400 for rabbit 2. On average, the titres obtained for the recombinant construct 3 protein (rabbits 1 and 2) were higher than those of recombinant construct 4 (rabbits 3 and 4). This may indicate that the larger construct 3 was more antigenic than the smaller construct 4, thus generating a higher immune response. In addition, the construct 3 protein could also contain extra epitopes.

Distribution studies carried out by immunoblotting revealed that almost all strains of bacteria tested reacted with the Omp87 recombinant protein antiserum. These results corresponded with the theory of Voulhoux *et al* (2003), who believe that homologues of the Omp87 protein exist in all gram negative organisms.

Almost all of the *L. pneumophila* serogroups 1-13 contained proteins of the expected size of 87 kDa, that reacted with the antiserum. Most of the other *Legionella* species tested also reacted with the antiserum, although the banding pattern obtained was different. Within the other *Legionella* species, and other bacterial species, there appeared to be some similarity with the banding patterns. In particular, most *Legionella* species, and several other bacterial species possessed a reactive protein of around 50 kDa, along with several smaller proteins. This may be due to protein degradation or breakdown, which resulted in smaller antigenic fragments which could still be recognised by the antibodies. If this was the case however, we would probably also expect the same for *L. pneumophila* serogroups 1-13, as all samples were processed in the same manner, and under the same conditions. Different protein sensitivity in different organisms, or different proteases or amounts of proteases, may however account for this observation (Bollag *et al*, 1996). The Omp87 protein of these organisms may not be as stable, or may be more prone to degradation by temperature or protease activity than the *L. pneumophila* Omp87. In addition, it may be possible that the Omp87 protein of these other *Legionella* and bacterial species possess a quaternary structure, and may be present, for example, in a dimer configuration. The protein would therefore separate into its individual components when subjected to electrophoresis under denaturing conditions, due to the reduction of disulphide bonds. Alternately, the reactivity seen with

the other *Legionella* and bacterial species may be due to cross-reactivity with a protein which is totally unrelated to the Omp87 of *L. pneumophila*.

The fluorescence microscopy study did not provide much insight into the location of the Omp87 protein in *L. pneumophila.* The control samples all reacted as expected. The *S. epidermidis* negative control did not reveal any binding with the polyclonal Omp87 antiserum, whilst the positive controls, such as the purified recombinant protein, revealed a great deal of binding. The whole cell *L. pneumophila* antiserum also reacted with samples, although to a lesser extent.

The fact that the polyclonal Omp87 antiserum did not react with *L. pneumophila* does not necessarily indicate that the Omp87 is not surface exposed. It may be because the serum was raised against a *denatured* form of the protein. Alternately, it may be that the β-barrel architectural structure typical of outer membrane proteins contains regions of differing antigenicity, just as it contains regions of differing chemical properties. These proteins usually have a tertiary structure which consists of a strongly hydrophobic barrel outer surface, and an amphipathic region at the membrane-solvent interface (Schulz, 2000). The Omp85 family of proteins are also believed to possess two domains. One is an NH_2-terminal periplasmic domain, and the other is a COOH-terminal "surface antigen" domain (Manning *et al*, 1998). Antibodies reacting with the surface antigen domain were found to be protective against *H. influenzae* infections in animal models (Loosmore *et al*, 1997). In *L. pneumophila*, however, this surface antigen domain may not be immunogenic, and may therefore not generate reactive antibodies.

As *Legionella* are gram negative organisms, they will also contain a leaflet of LPS surrounding the cell. These LPS molecules may also be interfering with the binding of antibodies to the Omp87 protein. It may therefore be a conformational difficulty in which the antibodies cannot readily access the protein which may result in the lack of antigen/antibody binding.

Further testing of the sensitivity and specificity of the Omp87 protein is required in order to gain a better understanding of the usefulness of the protein in diagnostics and detection systems. A wider scope of organisms, including all known *Legionella* species, should also be included in cross-reactivity testing, in order to fully determine the extent of Omp87 cross-reactivity within the genus *Legionella*, and with other unrelated organisms.

CHAPTER VIII

General Discussion

8. Introduction

Legionella spp., and *L. pneumophila* in particular, continue to be problematic worldwide, and potentially pose a threat to the health of the community. This is despite today's greater awareness for the need of more stringent guidelines for *Legionella* spp. control and prevention.

Outbreaks of legionellosis are often large and result in significant morbidity and mortality. The largest occurring in Australia to date being at the Melbourne aquarium in 2000. This outbreak resulted in 119 confirmed cases of Legionnaires' disease, and four deaths. The outbreak led to regulatory changes in Australia regarding *Legionella* control and prevention measures, and although these changes may have curbed the number of disease outbreaks, sporadic cases of disease continue to occur (O' Keefe *et al*, 2005). Worldwide, the largest reported outbreak of Legionellosis was in 1999, at the Westfriese Flora Show in the Netherlands. Here, 21 of the 231 people infected with *Legionella* succumbed to the disease (Wijgergangs, 1999). It is therefore clear that *Legionella* spp. have the potential to be highly detrimental to human health and well being.

Continuous monitoring of man-made environments in which *Legionella* spp. thrive would seem to provide a possible solution to the ongoing problem of both large and small scale outbreaks of Legionnaires' disease. Cooling towers and air-conditioning systems are examples of problematic areas which are often the source of disease outbreaks (Brown *et al*, 2001), and are areas for which the continuous monitoring of bacterial growth may prove advantageous.

Using biological sensors, or biosensors, is one approach which could be used for monitoring of environmental conditions. These could be in the form of a platform on which antigen-antibody binding kinetics is monitored and relayed through to an electronic detection and warning system. In order to construct the biological foundation of such a system, the appropriate antigen and antibody capture system must be devised which will be both sensitive and specific. In this thesis, an attempt was made to discover novel *L. pneumophila* outer membrane proteins which could potentially serve this purpose.

8.1 Identification of *L. pneumophila* Omp87

Through the use of both bioinformatics and laboratory techniques such as SDS-PAGE and MALDI-TOF Mass spectrometry, the *L. pneumophila* Omp87 protein was identified (chapter 3).

The use of bioinformatics tools has, in recent years, become a popular method for a diverse range of both genomic and proteomic investigations, such as the identification of novel genes or proteins, predictions of the structure and function of proteins, and many other DNA or protein sequence-related operations (Fickett, 1996). Although the task of searching databases for sequences or motifs can be time consuming, it is nonetheless an inexpensive, rapid, and less laborious option to pursue when compared to conventional laboratory methods, which often require expensive equipment or chemical reagents, and can require days, weeks or even months of experimentation. It can also prove to be indispensable in cases where the organism being investigated is difficult to culture or cannot be cultured in the laboratory.

In addition to the Omp87 protein, the bioinformatics protein motif sequence searching revealed many *L. pneumophila* membrane proteins, which included proteins that have not been previously described in *Legionella*. For the purpose of this study, only proteins in the outer membrane of *L. pneumophila* were considered. Proteins situated in the outer membrane of the cell are most often the focus of diagnostic or detection systems, as they are usually the first proteins which come into contact with the target antibody (Palmer, 2002).

Most of the protein matches which arose during the motif searching were not isolated to the outer membrane of the bacterial cell, and instead consisted mostly of enzymes which perhaps performed catalytic functions in the cell's outer membrane. These were therefore not useful for the purpose of this study.

During the laboratory process of analysing *L. pneumophila* outer membrane proteins (Chapter 3), a comparison of four different methods for the extraction of outer membrane proteins was performed. Two of the methods were similar, and involved similar completion times. The glycine-acid extraction method however, was different to the other methods and was the simplest and most rapid to perform. This method also gave results which were comparable to the other methods, and was therefore selected for

use with the analysis of *L. pneumophila* outer membrane proteins, and ultimately, the discovery of the Omp87 protein. This method of outer membrane protein extraction is not used routinely for *Legionella*, but is a commonly used technique with the enteric bacterium, *Campylobacter jejuni* (Garvis *et al*, 1996).

In addition to the Omp87 protein, the MALDI-TOF Mass spectrometry performed on the *L. pneumophila* outer membrane preparation (Chapter 3) also revealed a match with an outer membrane protein of the closely related *Xanthomonas axonopodis.* The protein match had a highly significant 'score' value of 241, and revealed a protein of 39 kDa. This protein appeared uncharacterised in *L. pneumophila*, and would therefore also be an interesting candidate for further analysis. Literature searches on outer membrane proteins of *Xanthomonas axonopodis pv. citri* did not reveal any publications on a 39 kDa protein, so no speculation can be made regarding the possible function of this protein.

Following the identification of the *L. pneumophila* Omp87, further analysis and characterisation of the protein was performed.

8.2 Characterisation of *L. pneumophila* Omp87

The *L. pneumophila* Omp87 protein was analysed following its discovery, in order to gain a better understanding of the protein, and to determine whether the protein was a possible candidate for use with a *Legionella* biosensor-based detection system.
The *omp87* gene was amplified from the *L. pneumophila* strain AA100 using novel PCR primers, which were designed based on the publicly available genome sequence of the *L. pneumophila* Philadelphia 1 strain. DNA sequencing was then performed, which revealed a sequence that was 98% identical to the Philadelphia 1 strain *omp87* gene.
It also revealed that the Omp87 protein was 786 amino acids in size (87 kDa), and contained a secretory signal sequence of around 44 amino acids in length, situated at the N-terminal region of the protein. These results were found to be very similar to the Omp87-like proteins of other organisms, such as the Omp85 of *N. gonorrhoea* and *N. meningitidis*, which were found to be 792 amino acids in length (Manning *et al,* 1998),

and the D15 outer membrane protein of *H. influenzae*, which is 797 amino acids in length (Flack *et al*, 1995).

PCR and Southern blotting was performed to determine whether other strains and serogroups of *Legionella* also contained the *omp87* gene. The same primer pair that was used for the PCR was also used to synthesise a probe for Southern blotting, and amplified part of the *L. pneumophila omp87* gene. *L. pneumophila* serogroups 1-13 were included in the PCR and Southern blotting experiments, in addition to 12 other *Legionella* species.

The PCR reaction amplified the *omp87* gene in all strains except *L. pneumophila* serogroups 3, 4, 5 and 8. We would however, expect the gene to be present in all serogroups. Nevertheless, this result was not surprising as the PCR is a very sensitive technique, and small variations in sequence can result in negative reactions due to the inability of the primer to bind completely to the DNA template. The fact that all of the strains were subjected to the same PCR conditions may also have been another factor affecting the results. Repetition of PCR negative serogroups, with optimisation of the PCR conditions for each individual serogroup may have resulted in PCR products for more strains.

The Southern blotting, which is less sensitive than the PCR, showed that the *omp87* gene was present in almost all serogroups of *L. pneumophila*. Only the serogroup 3 strain did not react with the probe, indicating that this serogroup may warrant repeat testing, perhaps using a different strain of the organism, particularly as this strain was also negative by PCR.

Mutagenesis studies were then performed on the *omp87* gene of *L. pneumophila*, in order to gain insight into the function of this protein. Studies with Omp87-like proteins in other organisms indicated that the protein is involved in the transport of either lipids or proteins to the outer membrane of the cell. In *Neisseria meningitidis*, depletion of the Omp85 protein resulted in a reduction of lipopolysaccharides and phospholipids in the outer membrane of the bacterial cell, and an accumulation of these lipids in the inner membrane of the cell (Genevrois *et al,* 2003).

Voulhoux *et al* (2003) however, believed that the Omp85 of *N. meningitidis* is involved in the insertion of proteins into the bacterial outer membrane, and that the decrease in

LPS observed by Genevrois *et al* was probably due to a defect in LPS transport machinery.

The *omp87* gene of *L. pneumophila* was disrupted by insertional inactivation with a kanamycin resistance gene. Natural transformation was then attempted to introduce the disrupted gene into the *L. pneumophila* genome, which was not successful. Possibly, the strain of *L. pneumophila* used in this study (FW02/001) did not possess the receptor molecules or recognition sequence necessary for the uptake of DNA through natural transformation (Smith, 1980).

Electroporation was therefore used to introduce the disrupted gene into the *L. pneumophila* genome. The disrupted gene was amplified by PCR, and these amplicons were introduced into *L. pneumophila* using electrotransformation. Homologous recombination between the functional *omp87* gene and the disrupted *omp87* gene was then believed to take place, via a double cross-over event. When the transformation mixture was plated on *Legionella* BCYE-α plates containing kanamycin, no transformants were obtained. As all of the control plates gave the expected results, we were able to speculate that the *omp87* gene is essential in *L. pneumophila*, and that its inactivation is lethal to cells. To confirm that the inactivation of the *omp87* gene is lethal and that the results obtained were not due to an inability of the DNA to enter cells, or the inability to recombine with the host's genome, a PCR reaction was performed. This was based on the method described by Burns *et al* (2000). The experiment was designed so that a product would only be obtained if integration of the disrupted gene had occurred with the *L. pneumophila* genome. As a product of the anticipated size was obtained we were able to conclude that the *omp87* gene was indeed an essential *L. pneumophila* gene. DNA sequencing of the product further confirmed this.

Additional studies are needed to further analyse the function of the *omp87* gene. Regulation of the amount of expression of the Omp87 protein through the use of inducible promoters or genes cloned upstream of the *omp87* gene would allow investigators to analyse the function of the protein without compromising the cells viability (Genevrois *et al*, 2003).

The final analysis performed on the *L. pneumophila* Omp87 involved the production of polyclonal antiserum against the protein (chapter 7). This was carried out using New Zealand White rabbits, over a 12 week period.

Pre-existing *E. coli* antibodies present in the pre-immune rabbit sera displayed reactivity with several *L. pneumophila* proteins. This cross-reactivity was not surprising as *E. coli*

and *Legionella* spp. are phylogenetically related. These *E. coli* antibodies were therefore removed by absorption of the sera with *E. coli* whole cells and cell lysates.
The antibody titre in the post-immune sera was determined by ELISA, and all rabbits showed a good level of antibody production. The titres ranged from 25,600 for rabbit 3, and were as high as 102,400 for rabbit 2. On average, the antibody production of rabbits 1 and 2 (construct 3) were higher than those of rabbits 3 and 4 (construct 4). This may be due to the larger construct 3 being more antigenic than the smaller construct 4. This could then result in the generation of a higher immune response.

Cross-reactivity studies were then performed using the immune sera to determine whether different serogroups and species of *Legionella*, in addition to several other related bacteria, were reactive towards the *L. pneumophila* Omp87 antiserum. The results showed that almost all strains of bacteria tested reacted with the *L. pneumophila* Omp87 antiserum. Variations in the reactive banding pattern were observed, but there were also similarities. *L. pneumophila* serogroups 1-13, in particular, gave the clearest banding pattern, with almost all strains displaying a band at around 87 kDa. Similarities were also observed with the banding pattern of other bacterial strains tested, whereby a 50 kDa protein, along with several smaller proteins reacted with the antiserum for most of the strains. This was probably due to protein degradation or breakdown, which resulted in smaller antigenic protein fragments.

Fluorescence microscopy carried out with *L. pneumophila* whole cells, the Omp87 polyclonal antiserum and secondary FITC-labelled anti-rabbit antiserum did not reveal the location of the Omp87 protein. As whole cells were used, reactivity with the antiserum would indicate that the protein was surface expressed, and able to be accessed by the antibodies in the antiserum. As the control samples gave the expected results, we speculated that the protein was not surface exposed, did not possess antigenic surface exposed regions, or was shielded by other molecules in the outer membrane.

8.3 Potential of the Omp87 for use in a *Legionella* detection system

This study found that the *L. pneumophila omp87* gene is most likely to be essential for survival of this organism. Essential genes are often considered to be ideal candidates for

bacterial detection systems. They usually are highly conserved, and undergo very little modifications. They therefore often share very similar DNA sequences between serogroups and species. This would lead us to speculate that the *L. pneumophila omp87* gene would be considered as a potential candidate for a detection system.

However, based on the analysis performed on the *L. pneumophila* Omp87 protein, it would appear that antibodies against the Omp87 protein would have limited use as a capture protein for a *Legionella* biosensor. Not only did the protein show cross-reactivity with many *Legionella* serogroups and species tested, but it was also cross-reactive with proteins from related bacterial species. Therefore, if polyclonal *L. pneumophila* Omp87 antibodies would be used in an antibody-based detection system, many false positive reactions would be obtained, which is obviously not desirable.

If cross-reactivity of the Omp87 protein was limited to *Legionella* serogroups and species, the protein would still be useful as a primary capture molecule. A second, more specific antigen or antibody could then be included in the system to differentiate between the species of *Legionella* present, once a positive reaction was obtained. As the *L. pneumophila* Omp87 also reacted with other bacterial species however, it could not be used in such a system.

There are however, several options which could possibly still enable the Omp87 to be used in a *Legionella* detection system. Firstly, a system using monoclonal Omp87 antibodies could greatly improve the specificity for the protein. Monoclonal antibodies are directed at certain epitopes of a protein and are therefore more specific than polyclonal antibodies, which are indiscriminately produced against many of the proteins epitopes. As expected, the extent of any cross-reactivity with the monoclonal antibodies would also need to be ascertained to determine the potential of the antibodies for use in the detection system. Secondly, if the cross-reactivity of the *L. pneumophila* Omp87 was restricted to other bacterial species which are related to *Legionella*, such as within the class of gamma Proteobacteria, absorbing the Omp87 antiserum with whole cell lysates, and outer membrane preparations of these organisms to remove the cross-reactive antibodies would also improve the specificity of the serum. Testing the range of organisms which cross-react with the Omp87 antiserum however, would be a laborious task, as a large and comprehensive collection of organisms would need to be tested in order to fully rule out any cross-reactivity with other unrelated organisms.

8.4 Future Directions

Clinical and environmental biosensors which rely on antigen/antibody or DNA/DNA hybridisation reactions are becoming increasingly prevalent. DNA-based systems in particular, are generally regarded as highly specific and sensitive, such as the use of PCR-based detection systems. Previous work in which I was involved aimed at the environmental field testing of samples, and the miniaturisation of PCR machines to enable on-site PCR testing of environmental samples (Ivanova *et al*, 2002). Today however, conventional PCR-based systems appear to be gradually declining in popularity in favour of real-time PCR methods and high throughput systems such as microarray-based systems, which are also highly sensitive and specific. Although antigen/antibody based systems have been in widespread use for numerous years, the obvious advantages of DNA microarray systems, such as the ability to test numerous samples simultaneously for the presence of many different genes (Zhou, 2003) means that these systems are likely to surpass the use of current methods.

In the context of DNA-based systems, the *omp87* gene may prove useful as an indicator for the presence of *Legionella* spp. in environmental or clinical samples. The gene sequence of the *omp87* gene could be analysed for the presence of unique signature sequences which are exclusive to *Legionella*, or even to *L. pneumophila*, thereby greatly enhancing the specificity of the gene. These DNA signature sequences could then be used in a microarray type detection system which could process numerous samples simultaneously, or could include several *Legionella* genes which would enable for typing of the organism. In a clinical setting, the *L. pneumophila omp87* gene could be included in a test to diagnose patients suspected of having a lung infection. Representative genes of other organisms commonly implicated in lung infections could also be included, and a complete test could be devised which, with one sample of sputum or excised lung cells (biopsy) could provide a diagnosis for the cause of the infection.

Despite large scale media attention, and increased public awareness of the dangers of *Legionella* and the symptoms of disease, outbreaks of Legionnaires' disease continue to occur with a somewhat regular prevalence. Therefore, whether future *Legionella* detection systems are antigen/antibody or DNA/DNA based, it is clear that an effective,

cost-efficient and user friendly system needs to be devised which can be routinely implemented by large and small corporations alike, into their building's water systems. With the assistance of government regulations to ensure that these systems remain in place, we would hope that the incidence of Legionnaires' disease outbreaks in the community receded to the point of becoming a rarity.

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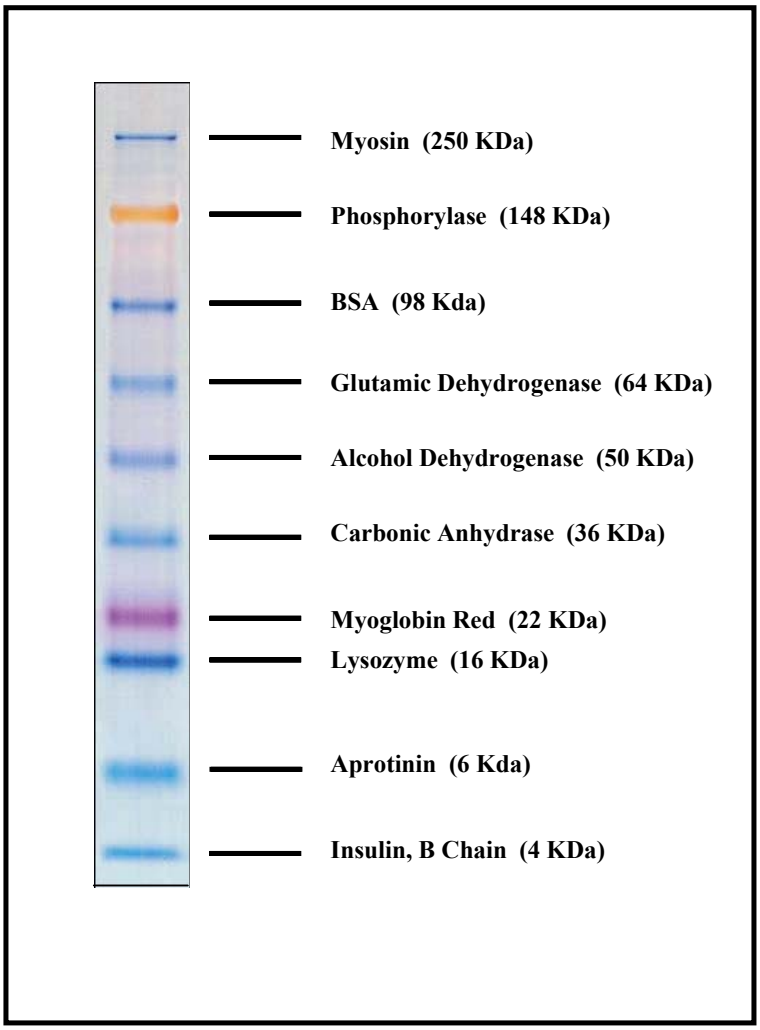
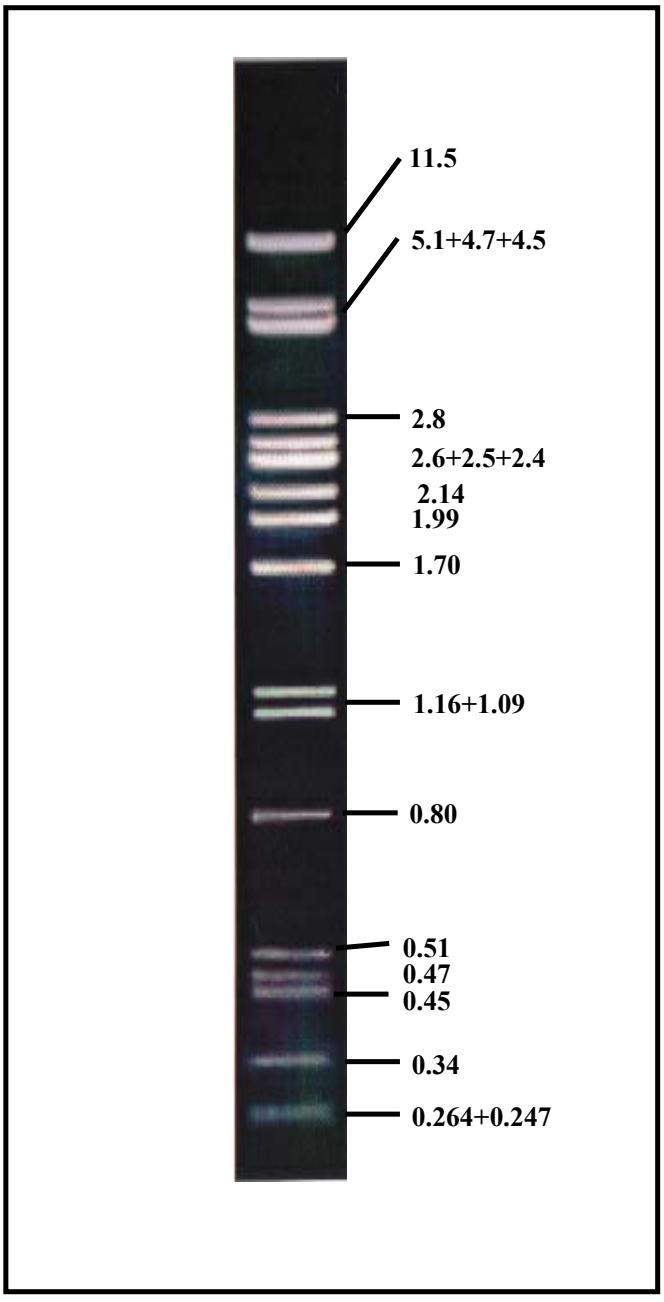
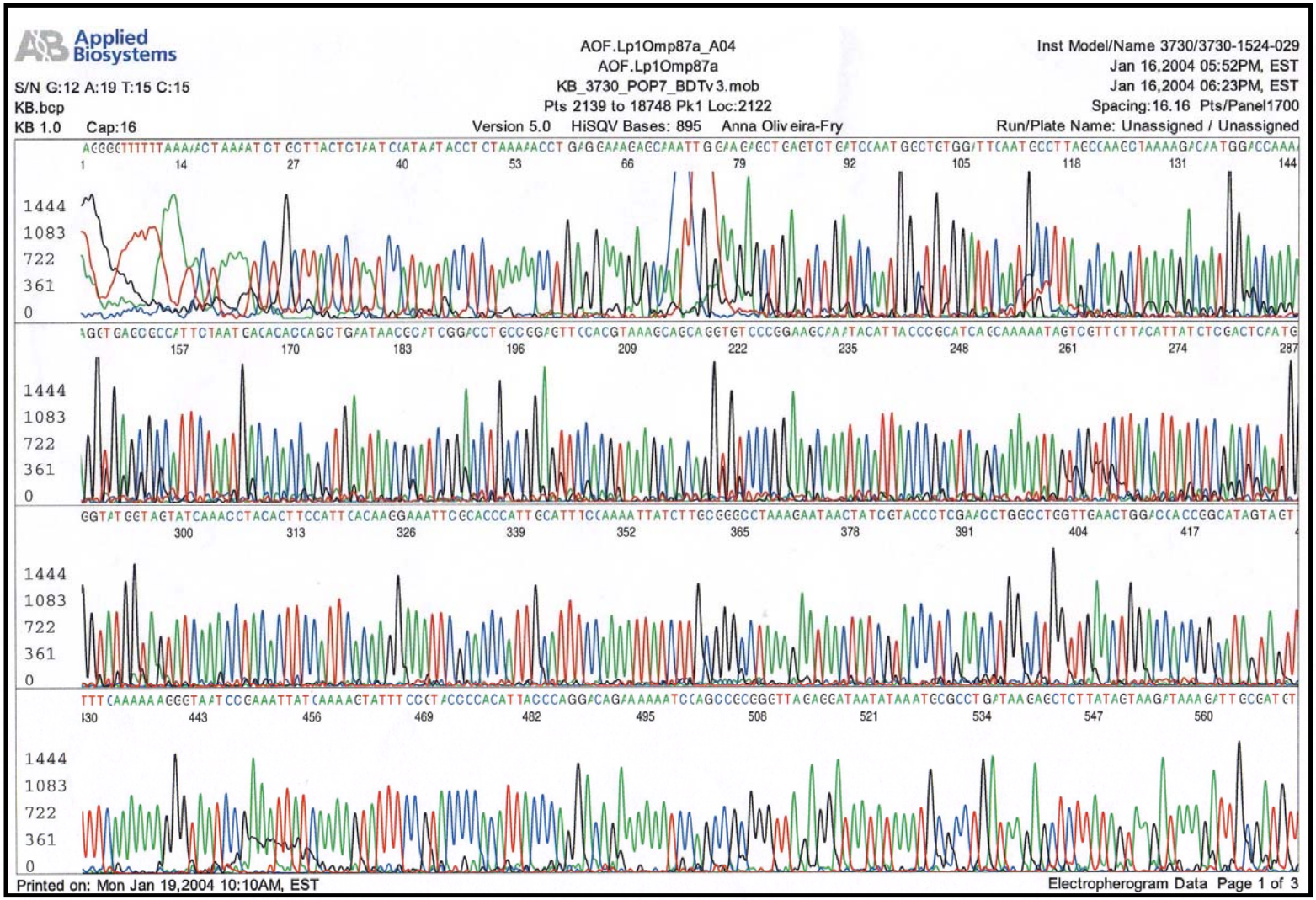
Appendix 1: SeeBlue ™ Plus2 Pre-Stain Molecular weight standard



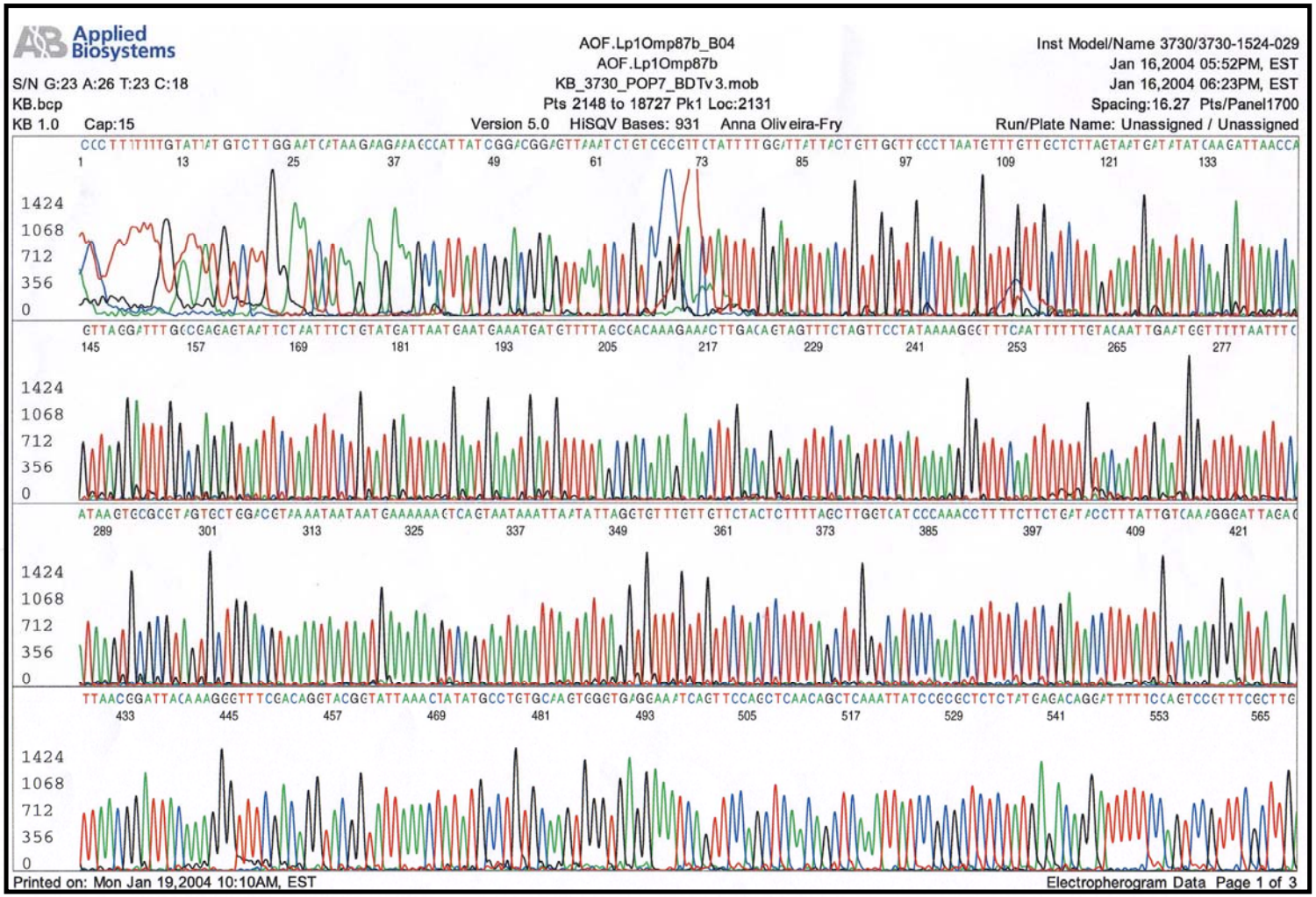
Appendix 2: λ x *Pst*1 Molecular weight marker



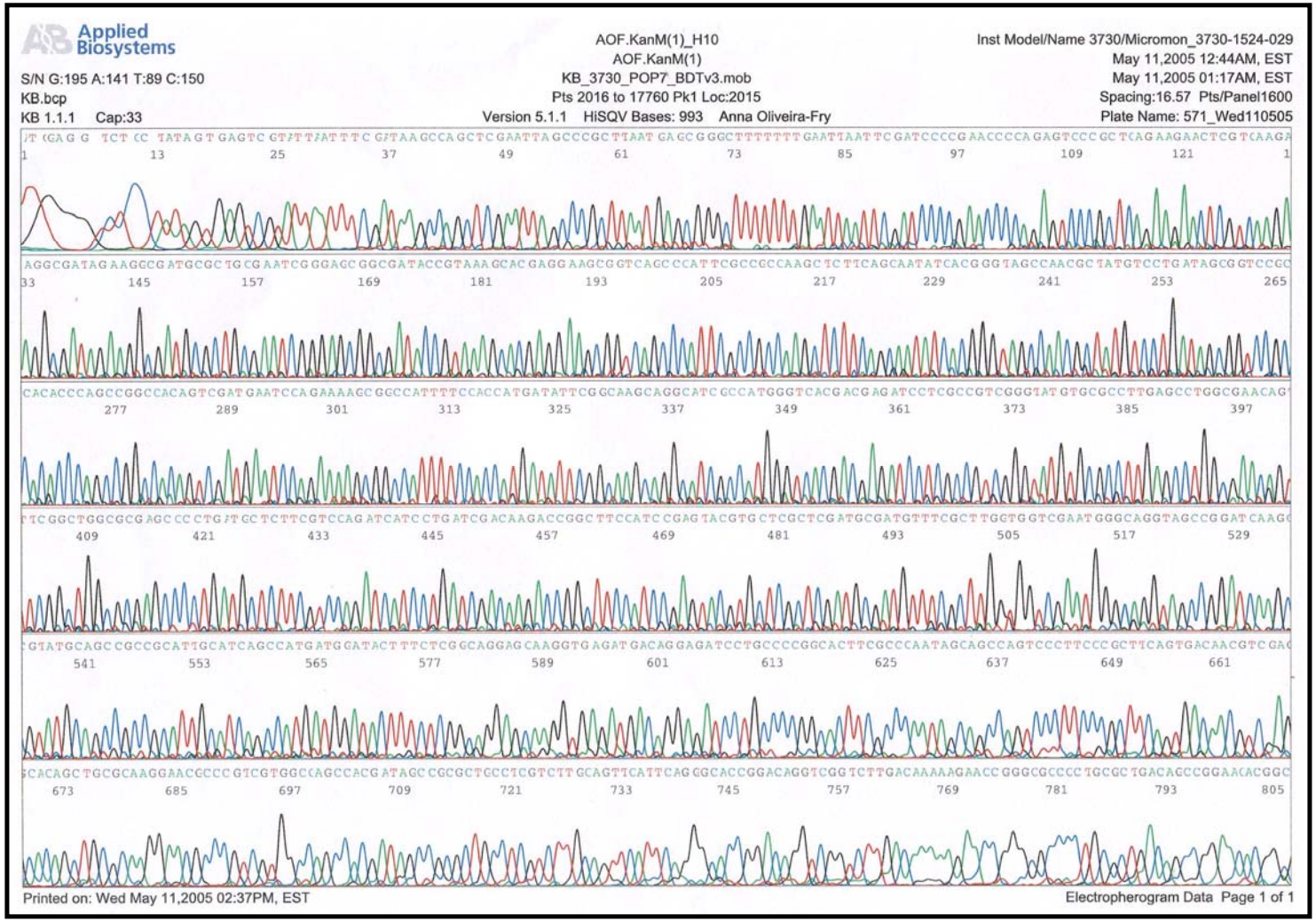
Appendix 3A: DNA sequencing chromatogram of *omp87* gene (a)



Appendix 3B: DNA sequencing chromatogram of *omp87* gene (b)



Appendix 4: DNA sequencing chromatogram of inactivated *omp87* gene (AOFIAKanB)



Appendix 5: Titration results (in triplicate) of ELISA assay for Rabbits 1-4.

The titrations were performed in triplicate for each rabbit, and the average of these 3 titrations were calculated. The background OD_{450nm} was subtracted from the average, and these values were plotted on the graphs shown in chapter 7, Figures 7.4 (a-d).

Rabbit 1	1.358	1.289	1.276	1.27	1.295	1.227	1.262	1.262	1.223	1.01	0.66	0.419
	1.332	1.264	1.252	1.276	1.264	1.305	1.219	1.198	1.156	0.954	0.659	0.412
	1.223	1.239	1.314	1.224	1.239	1.183	1.217	1.152	1.031	0.832	0.578	0.377
Average	1.304	1.264	1.281	1.257	1.266	1.238	1.233	1.204	1.137	0.932	0.632	0.403
-Background	**0.659**	**0.619**	**0.636**	**0.612**	**0.621**	**0.593**	**0.588**	**0.559**	**0.492**	**0.287**	**-0.013**	**-0.242**
Rabbit 2	1.29	1.297	1.291	1.285	1.298	1.258	1.258	1.258	1.18	0.996	0.711	0.434
	1.173	1.188	1.209	1.254	1.199	1.158	1.172	1.153	1.121	0.938	0.667	0.427
	1.242	1.231	1.205	1.27	1.226	1.215	1.199	1.199	1.135	0.969	0.653	0.423
Average	1.235	1.239	1.235	1.270	1.241	1.210	1.210	1.203	1.145	0.968	0.677	0.428
-Background	**0.59**	**0.594**	**0.59**	**0.625**	**0.596**	**0.565**	**0.565**	**0.558**	**0.5**	**0.323**	**0.032**	**-0.217**
Rabbit 3	1.139	1.057	1.084	1.122	1.061	1.062	1.036	0.958	0.763	0.488	0.267	0.099
	1.127	1.02	1.051	1.022	1.08	1.032	1.02	0.911	0.799	0.494	0.315	0.131
	1.122	1.082	1.052	1.032	1.073	1.023	1.027	0.953	0.786	0.516	0.328	0.143
Average	1.129	1.053	1.062	1.059	1.071	1.039	1.028	0.941	0.783	0.499	0.303	0.124
-Background	**0.484**	**0.408**	**0.417**	**0.414**	**0.426**	**0.394**	**0.383**	**0.296**	**0.138**	**-0.146**	**-0.342**	**-0.521**
Rabbit 4	1.179	1.145	1.075	1.078	1.113	1.109	1.08	1.002	0.811	0.619	0.345	0.155
	1.106	1.078	1.006	1.013	1.01	0.984	0.999	0.905	0.755	0.453	0.321	0.147
	1.13	1.116	1.058	0.935	1.022	1.038	1.039	0.982	0.853	0.597	0.394	0.186
Average	1.138	1.113	1.046	1.009	1.048	1.044	1.039	0.963	0.806	0.556	0.353	0.163
Background	**0.493**	**0.468**	**0.401**	**0.364**	**0.403**	**0.399**	**0.394**	**0.318**	**0.161**	**0.079**	**-0.292**	**-0.482**